Mol Gen Genomics (2006)
DOI 10.1007/s00438-006-0115-y

ORIGINAL PAPER

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The *Rhizobium leguminosarum* regulator IrrA affects the transcription of a wide range of genes in response to Fe availability

Received: 8 November 2005 / Accepted: 20 February 2006
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Abstract We show that an unusual transcriptional regulator, called IrrA, regulates many genes in the symbiotic N_2-fixing bacterium *Rhizobium leguminosarum* in response to iron availability. Several operons in *R. leguminosarum* are expressed at lower levels in cells grown in Fe-depleted compared to Fe-replete medium. These include *hemA1*, which encodes the haem biosynthesis enzyme amino-levulinic acid synthase; *sufS2BCDS1XA*, which specify enzymes for FeS cluster synthesis; *rirA*, a global, Fe-responsive transcriptional repressor; *RL0400*, which likely encodes an unusual FeS cluster scaffold; and the possible ferri-siderophore ABC transporter *rrp1*. Reduced expression in Fe-depleted medium was effected by IrrA, a member of the Fur super-family, which in *Bradyrhizobium*, the symbiont of soybeans, and in the mammalian pathogen *Brucella*, is unstable in Fe-replete conditions, due to an interaction with haem. The *R. leguminosarum* IrrA likely interacts with ICE (iron-control element) motifs, conserved sequences near the promoters of its target genes. The *rirA*, *sufS2BCDS1XA* and *rrp1* genes are also known to be regulated by RirA, which represses their expression in Fe-replete medium. We present a possible model for iron-responsive gene regulation in *Rhizobium*, in which the IrrA and RirA regulators, working in parallel, respond to the intracellular availability of haem and, possibly, of FeS clusters respectively. Thus, these regulators may sense the physiological consequences of extraneous Fe concentrations, rather than the concentration of Fe per se, as happens in those bacteria (e.g. *Escherichia coli*) in which the ferric uptake regulator Fur is the global Fe-responsive gene regulator.

Electronic Supplementary Material Supplementary material is available for this article at http://dx.doi.org/10.1007/s00438-006-0115-y and is accessible for authorized users.

Communicated by A. Kondorosi

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Introduction

Rhizobia are symbiotic α-proteobacteria, with different biovars, species and genera inducing N_2-fixing nodules on the roots of many different legumes. These bacteria require iron, not only in the nodule (where the enzyme nitrogenase and various ancillary proteins needed for N_2 fixation have Fe in their cofactors), but also in the oligotrophic soils, where they must obtain Fe in competition with other organisms. It has been established that the Rhizobia can acquire Fe in many different ways, including siderophores (Fe-binding, chelating molecules), haem, ferric citrate and individual strains also have multiple ABC transporters that may import inorganic Fe (see Johnston 2004). It is also clear that the ways in which genes in these bacteria are regulated in response to iron availability are very different from those that have been described for model bacteria, such as *Escherichia coli*. In the present study, we describe an unusual Fe-responsive transcriptional regulator in *Rhizobium leguminosarum* and show that it affects the expression of several different types of structural genes.

In *R. leguminosarum* biovar *viciae*, which nodulates peas and vetches, the genes for the synthesis (*vbs*) and uptake (*fhu*) of the siderophore vicibactin, and those for the uptake of haem (*hmu*), as well as some of the ABC

transporter genes, are repressed in response to Fe, this being mediated by a transcriptional regulator termed RirA (Todd et al. 2002). Proteomic analyses (Todd et al. 2005) confirmed that RirA is a global regulator, since >80 proteins whose synthesis was normally repressed in Fe-replete (+Fe) conditions were synthesized constitutively in a RirA$^-$ mutant. Also, the expression of some other proteins was enhanced in +Fe conditions in the wild type but was deregulated in RirA$^-$ mutants (Todd et al. 2005). Similarly, mutations in *rirA* of the closely related *Sinorhizobium meliloti* (which nodulates alfalfa) abolish Fe-dependent repression of genes involved in Fe uptake (Viguier et al. 2005) and in other functions that impinge on Fe metabolism (Chao et al. 2005). In the *cis*-acting regulatory regions of many RirA-repressible genes is a conserved sequence, the iron-responsive element (IRO), the likely RirA-binding site (Yeoman et al. 2004).

Global control of gene expression in response to Fe availability is not in itself unexpected. What is unusual is the identity of the regulator, RirA, compared to other Fe-responsive gene regulators. Best known of these is Fur (ferric uptake regulator), which has been intensively studied in the γ-proteobacteria *E. coli* and *Pseudomonas aeruginosa* and in some Gram positive bacteria, including *Bacillus subtilis* (see Andrews et al. 2003). Fur, when complexed with Fe^{2+}, binds *cis*-acting *fur* boxes that precede many (>100) Fur-repressed genes, in at least some bacteria (Andrews et al. 2003). *E. coli* Fur also activates expression of some genes by a two-step process in which Fur represses *ryhB*, which encodes a small inhibitory RNA, preventing expression of several genes at a post-transcriptional level (see Gottesman 2004). Another class of wide-ranging Fe-responsive regulators, found in Gram positive bacteria, includes DtxR, whose structure resembles that of Fur, despite a lack of amino acid sequence similarity (de Peredo et al. 2001). *R. leguminosarum* and *S. meliloti* both have a Fur homologue, but it has a different, minor, role compared to conventional Fur. It is a manganese-responsive regulator, termed Mur, which in Mn^{2+}-replete media represses expression of the Mn^{2+} uptake *sitABCD* operon by binding to *cis*-acting regulatory MRS (manganese-responsive sequence) boxes (Chao et al. 2004; Diaz-Mireles et al. 2004, 2005; Platero et al. 2004).

The sequence of RirA is wholly different from Fur and DtxR, and IRO motifs have no similarity to canonical *fur* boxes. RirA is in the large (>350 members) and taxonomically widespread Rrf2 super-family, only three other members of which have been examined. These are Rrf2 of *Desulfovibrio* (Keon et al. 1997), NsrR in *Nitrosomonas* (Beaumont et al. 2004), and IscR of *E. coli*, which, respectively regulate genes that specify cytochromes, nitrite reductase and the Isc proteins involved in FeS cluster synthesis. IscR can form reversible complexes with FeS clusters and, in its FeS-IscR holo form, binds tightly to the *isc* promoter, repressing its transcription (Schwartz et al. 2001). Close homologues (~70% identity) of RirA only occur in some genera of the order Rhizobiales, namely, *Sinorhizobium, Mesorhizobium*, the mammalian pathogens *Brucella* and *Bartonella*, the phytopathogen *Agrobacterium*, but not, perhaps surprisingly, the more distantly related *Bradyrhizobium japonicum*.

Another Fe-responsive regulator, Irr, was identified in *B. japonicum*, which nodulates soybeans (Hamza et al. 1998). Irr is in the Fur super-family, the Irr branch being confined to α-proteobacteria, some genera of which (*Bradyrhizobium, Brucella, Rhizobium*) have two versions of this gene (see Fig. 1). Irr of *B. japonicum* (Irr_{Bj}) represses *hemB*, which encodes δ-aminolevulinic acid dehydratase, the second catalytic step in haem biosynthesis. This repression occurs only in -Fe conditions; in +Fe media, Irr is degraded through an interaction between Irr and haem (Qi and O'Brian 2002; Yang et al. 2005). The interaction between haem and Irr_{Bj} occurs at two different regions of the protein. One of these, at its N-terminus (Qi and O'Brian 2002) resembles a haem regulatory motif (HRM), which is involved in various haem–protein interactions, in pro- and eukaryotes (Zhang and Guarente, 1995). The second region is at the C-terminal part of Irr_{Bj} and includes three consecutive His residues (Yang et al. 2005).

Recently, the Irr protein of the pathogen *Brucella abortus* was shown to be involved in regulating haem synthesis and, as in *B. japonicum*, to exhibit post-translational instability in cells grown in Fe-replete media (Martinez et al. 2005). We had shown (Wexler et al. 2003) that an Irr$^-$ mutant of *R. leguminosarum* was deregulated for haem biosynthesis, although the exact target gene(s) were not established. We show here that one of the Irr proteins of *R. leguminosarum* (IrrA) has a wider role, regulating several genes in response to Fe availability. These data, together with previous observations on the RirA regulator, lead us to propose a model for Fe homeostasis in *Rhizobium* which differs markedly from that which applies to *E. coli*.

Materials and methods

Strains and plasmids

These are shown in Table 1.

Media, bacterial growth conditions and in vivo genetic manipulations

Bacteria were grown as in Beringer (1974). In high-Fe medium, $FeCl_3$ (20 μM) was added; low-Fe medium had no added Fe, but 20 μM of the Fe chelator 2,2-di-pyridyl was present. Plasmids were conjugated into *R. leguminosarum* using helper plasmid pRK2013 (Figurski and Helinski 1979). β-galactosidase assays were done as in Rossen et al. (1985).

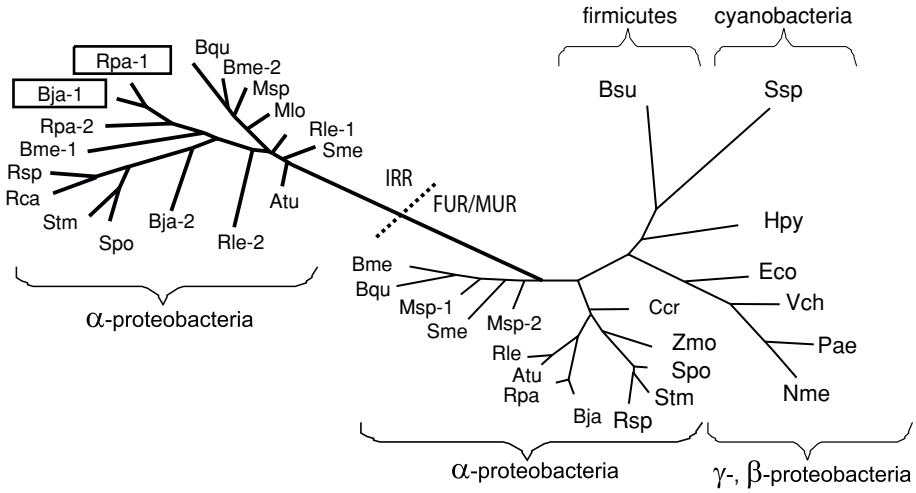

Fig. 1 Phylogenetic tree of Irr and Fur/Mur regulators from α-proteobacteria and Fur regulators in other species. The separate branch containing Irr proteins in α-proteobacteria is shown by *bold lines*. Experimentally characterized Fur regulators are from *Bacillus subtilis* (Bsu), *Escherichia coli* (Eco), *Synechococcus* sp. (Ssp), *Helicobacter pylori* (Hpy), *Vibrio cholerae* (Vch), *Pseudomonas aeruginosa* (Pae), and *Neisseria meningitidis* (Nme). Ccr and Zmo stand for *Caulobacter crescentus* and *Zymomonas mobilis*, respectively. The genomic abbreviations of the other α-proteobacteria are as listed in the Materials and methods, and Irr proteins with a haem regulatory motif (HRM) at their N-termini are boxed. Genomic identifiers of Irr proteins are RL0115 (Rle-1)), pRL110146 (Rle-2), SMc00329 (Sme), AGR C 249 (Atu), mlr5570 (Mlo), MBNC03003186 (Msp), BQ01230 (Bqu), BMEI1563 (Bme-1), BMEI1955 (Bme-2), blr0768 (Bja-1), blr1216 (Bja-2), Rsph03001693 (Rsp), STM1w01001534 (Stm), SPOA0445 (Spo), RPA0424 (Rpa-1), RPA2339 (Rpa-2). Genomic identifiers of Fur/Mur proteins in α-proteobacteria are MBNC03003179 (Msp-1), MBNC03003593 (Msp-2), SMc02510 (Sme), BQ01860 (Bqu), BMEI0375 (Bme), RPA0450 (Rpa), bll0797 (Bja), CC0057 (Ccr), ZM01412 (Zmo), Rsph03000505 (Rsp), STM1w01000993 (Stm), SPO2477 (Spo), AGR C 620 (Atu), RL0397 (Rle). The dotted line acts to distinguish the Irr and the Fur/Mur branches of the Fur super-family

In vitro DNA manipulations

Routine DNA manipulations were done essentially as in Wexler et al. (2001). *R. leguminosarum* genomic DNA was isolated using a Promega preparation kit. Promoter-containing fragments were made using *R. leguminosarum* genomic DNA as template and primers designed using the *R. leguminosarum* strain 3841 genome sequence (http://www.sanger.ac.uk/Projects/R_leguminosarum/). These primers contained appropriate restriction sites as shown in Table 2. The PCR products were then cloned into the wide host-range promoter-probe plasmid vector pMP220 (Spaink et al. 1987).

Table 1 Strains and plasmids used in this study

Bacteria	Properties	Source
E. coli		
803	Used as host for plasmids	Wood (1966)
E. coli DH10B-T1R	Used for site-directed mutations	Invitrogen
R. leguminosarum		
J251	Wild type; Str^r	Wexler et al. (2001)
J386	J251 *irrA*::Tn*5lac*	Wexler et al. (2003)
J397	J251 *rirA*::Tn*5*	Todd et al. (2002)
Plasmids		
pMP220	Wide host-range promoter probe; Tet^r	Spaink et al. (1987)
pRK2013	Used as helper in triparental conjugations	Figurski and Helinski (1979)
pBIO1580	*hemA1-lacZ* fusion in pMP220	This work
PBIO1581	*hemA2-lacZ* fusion in pMP220	This work
pBIO1582	*hemB-lacZ* fusion in pMP220	This work
pBIO1439	*rirA-lacZ* fusion in pMP220	Todd et al. (2002)
pBIO1497	*suf-lacZ* fusion in pMP220	Todd et al. (2005)
pBIO1553	*RL0400-lacZ* fusion in pMP220	This work
pBIO1558	*rrp1-lacZ*	This work
pBIO1586	Contains promoter of *sufS2BCDS1XA* with three site directed mutations in the ICE motif cloned in pMP220	This work
pBIO1587	Contains promoter of *sufS2BCDS1XA* with six site directed mutations in the ICE motif cloned in pMP220	This work

Table 2 Oligonucleotides used in this study

Name	Sequence	Comments
RL0400p1	GGAATTCCGCGAGGGCGGTGCTGC	Amplifies RL0400 promoter
RL0400p2	AACTGCAGCCGGCTTAAGATGCTGC	Amplifies RL0400 promoter
RL0400PE	GCGGGGTTCGGCGTGGCTTCGG	Used for RL0400 primer extension
hemA1p1	GTGGGATCCTGGCCCATGCCGAGATAGTC	Amplifies *hemA1* promoter
hemA1p2	GGAATTCGCCGCTCGCATATTCTTTATC	Amplifies *hemA1* promoter
hemA1PE	CGGTGTGAAGCCCGTCCAGCGCGC	Used for *hemA1* primer extension
hemA2p1	GGAATTCTCAAGCTAGCGGCACTGC	Amplifies *hemA2* promoter
hemA2p2	AACTGCAGATCAGTGCCGCTTCCTTGC	Amplifies *hemA2* promoter
hemBp1	GGAATTCCATAGCGCAGACATCGG	Amplifies *hemB* promoter
hemBp2	AACTGCAGTCGAGATAGGGCAGGCCGGGC	Amplifies *hemB* promoter
rrp1p1xba1	GCTCTAGATGCGAGATGACGGCGGTGC	Amplifies *rrp1* promoter
rrp1p2xba1	GCTCTAGAGCCGGTGACGACGATGACG	Amplifies *rrp1* promoter
SufICE1	ccagtttagaacaaGCGgaaacttgcatggcgatcc	First round of mutagenesis of the *suf* ICE motif
SufICE2	gatcgccatgcaagtttcCGCttgttctaaactgcg	First round of mutagenesis of the *suf* ICE motif
SufICE3	cgcagtttagaacaaGCGgGGGcttgcatggcgatcc	Second round of mutagenesis of *suf* ICE motif
SufICE4	ggatcgccatgcaagCCCcCGCttgttctaaactgcg	Second round of mutagenesis of *suf* ICE motif

Restriction sites in the oligonucleotides are underlined as appropriate. For the two pairs of mutagenic primers, the altered bases are shown in capitals

Site-directed mutagenesis of the ICE box preceding *sufS2BCDS1XA* was generated using a QuikChange XL (Stratagene) mutagenic PCR kit according to the manufacturer's instructions. The template DNA was plasmid pBIO1497, which contains a 808 bp DNA fragment spanning the *suf* promoter and regulatory regions, cloned in pMP220. Mutagenic primers, SufICE1 and SufICE2, each 37 bp in length (Table 2) that spanned the *suf* ICE motif, contained 3 bp substitutions (GCG for TTC) compared to wild-type and were used in a mutagenic PCR reaction. The products were transformed into *E. coli* DH10B-T1R (Invitrogen), selecting tetracycline resistance and mutant plasmids were ratified by sequencing. One such verified mutated plasmid (termed pBIO1586) was then used as the template in a second round of mutagenesis using primers SufICE3 and SufICE4 (Table 2). These change the wild type sequence (AAA) to GGG. The resultant mutant plasmid, pBIO1587, therefore has mutations in six of the highly conserved nucleotides in the ICE motif.

RNA extractions from *R. leguminosarum*, primer extensions and DNA sequencing were done as previously described, to determine the transcriptional starts of *hemA1* and *RL0400* (Sawers and Böck 1989; Wexler et al. 2001). The oligonucleotides used are shown in Table 2.

Bioinformatic analyses

Sequence data of α-proteobacterial genomes

Complete genomes of *Agrobacterium tumefaciens* (Atu), *Bartonella quintana* (Bqu), *Bradyrhizobium japonicum* (Bja), *Brucella melitensis* (Bme), *Mesorhizobium loti* (Mlo), *Rhodopseudomonas palustris* (Rpa), *Silicibacter pomeroyi* (Spo), *Sinorhizobium meliloti* (Sme), and unfinished annotated genomic sequences of *Mesorhizobium* sp. BNC1 (Msp), *Rhodobacter sphaeroides* (Rsp), and *Silicibacter* sp. TM1040 (Stm) were downloaded from GenBank (Benson et al. 2005). Unpublished genomes of *Rhizobium leguminosarum* (Rle) and *Rhodobacter capsulatus* (Rca) were downloaded from the Web sites of the Wellcome Trust Sanger Institute (http://www.sanger.ac.uk) and Integrated Genomics Inc. (http://www.integratedgenomics.com), respectively.

Identification of regulatory signals

An iterative signal detection procedure implemented in the program SignalX (Gelfand et al. 2000) was used to identify a common regulatory DNA motif in the upstream regions of the IrrA-regulated genes, *hemA1*, *rirA* and *sufS2* in *R. leguminosarum*. This motif with consensus AnTTTRGAAYnRTTCYAAAnT (where R and Y are puRine and pYrimidine, respectively) was scanned against the genomes of α-proteobacteria using the GenomeExplorer software (see http://www.bioinform.genetika.ru/projects/reconstruction/index.htm), and additional genes with candidate ICE motifs in the upstream regions were selected (Table 1; Supplementary Table 1). Positional nucleotide weights in the recognition profile and Z scores of candidate sites were calculated as the sum of the respective positional nucleotide weights as described in Mironov et al. (1999). The threshold for the site search was defined as the lowest score observed in the training set.

Genomic analyses (protein similarity searches using the Smith–Waterman algorithm, analysis of orthology, and identification of candidate sites in genomic sequences) used Genome Explorer (Mironov et al. 2000). Multiple protein alignments were done using ClustalX (Thompson et al. 1997). Phylogenetic trees were created by the maximum likelihood method implemented in PHYLIP (Felsenstein 1981). Additional protein similarity searches were done using BLAST (McGinnis and

Madden 2004). Conserved protein domain annotations used the PFAM database (Bateman et al. 2002).

Results

Inspection of the genome sequence of *R. leguminosarum* strain 3841 revealed two genes whose deduced products were in the Irr branch of the Fur superfamily (Fig. 1). One of these, which we term *irrA*, is the chromosomal *RL0115* (shown as Rle-1 in Fig. 1) which is closely related to the version of Irr in *Brucella* described by Martinez et al. (2005). The other, which we term *irrB*, is on the large plasmid, pRL11, and has the identifier *pRL110146* (shown as Rle-2 in Fig. 1). We had shown that IrrA is involved in regulating haem synthesis in *R. leguminosarum*, so the following work focusses on this gene.

Irr of *B. japonicum* acts as a transcriptional repressor in cells grown in Fe-depleted media. We therefore investigate if one of the Irr's of *R. leguminosarum* was responsible for our previous findings that several genes in this species were transcribed at higher levels in +Fe than in −Fe media (Wexler et al. 2003; Todd et al. 2005) These included *hemA* (haem biosynthesis), *rirA* (iron-responsive regulator) and *sufS2BCDS1XA* (synthesis of FeS clusters). To do this, we made (or used pre-existing) reporter fusions in which the promoters of genes that displayed this pattern of regulation were cloned into the wide host-range reporter plasmid pMP220. The resultant constructs were mobilized into the IrrA$^-$ mutant strain J386 and, for comparison, into the wild type J251 and into the RirA$^-$ mutant J397. The transconjugants were assayed for β-galactosidase activity after growth in Fe-replete and Fe-depleted media. The results obtained with these assays are presented in the sections below.

IrrA regulates the haem biosynthetic gene *hemA1*, but not *hemA2* or *hemB*

Transcription of *R. leguminosarum hemA* is higher (~threefold) in +Fe compared to −Fe medium (Wexler et al. 2003). We now rename this gene (*RL4379*) *hemA1* because the *R. leguminosarum* strain 3841 genome (http://www.sanger.ac.uk/Projects/R_leguminosarum/) reveals two *hemA* genes, whose products are 99.5% identical to each other, with *hemA1* corresponding to the *hemA* that was studied by Wexler et al. (2003). The other, *hemA2* (*pRL90008*), is on the native plasmid pRL9. Three other α-proteobacteria, *M. loti, R. sphaeroides* and *S. pomeroyi*, also have two copies of *hemA* (Neidle and Kaplan 1993; our observations); perhaps surprisingly, *S. meliloti*, a close relative of *R. leguminosarum*, only has only one *hemA*, which is required for symbiotic N_2 fixation (Leong et al. 1982).

We constructed *hemA1-lacZ* and *hemA2-lacZ* plasmids (respectively pBIO1580 and pBIO1581) and mobilized them into wild type *R. leguminosarum* and into the J386 and J397 mutants. As anticipated, in the wild type and in J397, *hemA1-lacZ* was expressed at higher levels in +Fe medium, but in the IrrA$^-$ mutant, it was expressed at similar, relatively high levels in +Fe and −Fe media (Fig. 2a). In contrast, transcription of *hemA2-lacZ* was very low (~50 Miller Units), barely above the background level with the vector pMP220 alone (data not shown). This lack of expression was found in both +Fe and −Fe media and in the wild type, and the IrrA$^-$ and RirA$^-$ mutants (not shown). Other studies (J. D. Todd, unpublished) show that *hemA2* expression is subject to control by O_2 availability, being transcribed only when the cells are grown in microaerobic conditions.

The deregulated expression of *hemA1* in the *R. leguminosarum* IrrA$^-$ mutant would account for its accumulation of protoporphyrin IX (PPIX), the immediate precursor of haem (Wexler et al. 2003). *B. japonicum* Irr$^-$ mutants also accumulate PPIX, but by deregulation of *hemB*, not *hemA* (Hamza et al. 1998). We therefore examined if *R. leguminosarum* IrrA affected *R. leguminosarum hemB* (*RL1616*) expression by cloning its promoter region into pMP220, to form pBIO1582. Expression of this *hemB-lacZ* fusion was at relatively low levels (~150 Miller Units), irrespective of Fe availability in the medium or the *rirA* or *irrA* genotypes. Thus the regulation of the haem biosynthetic pathway differs in *R. leguminosarum* and *B. japonicum*, even though IrrA is a transcriptional regulator of at least one *hem* gene in both species.

Effect of a mutation in *irrA* on the expression of *rirA*

Expression of *rirA* is enhanced ~threefold in *R. leguminosarum* grown in +Fe compared to −Fe-media (Todd et al. 2002). This was confirmed here, using the *rirA-lacZ* fusion pBIO1439 (Fig. 2b). Todd et al. (2002) also found that RirA was auto-regulated, to some extent; in a RirA$^-$ mutant, expression of *rirA-lacZ* is further enhanced in +Fe medium. When the *rirA-lacZ* fusion plasmid was in the IrrA$^-$ mutant J386, the levels of β-galactosidase activities were significantly enhanced compared to the wild type background, but, importantly, this only occurred in the −Fe medium; in Fe-replete conditions, the *irrA* mutation had no discernible effect (Fig. 2b). RirA is therefore under dual control, being subject to auto-regulation under high-Fe conditions, and IrrA-dependent repression under low-Fe conditions.

The *sufS2BCDS1XA* operon is regulated by IrrA and by RirA

The behavior of the *rirA* fusions was reminiscent of previous observations on Fe-responsive regulation of the *R. leguminosarum sufS2BCDS1XA* operon (*RL2583-RL2577*), which encodes the enzymes needed for the

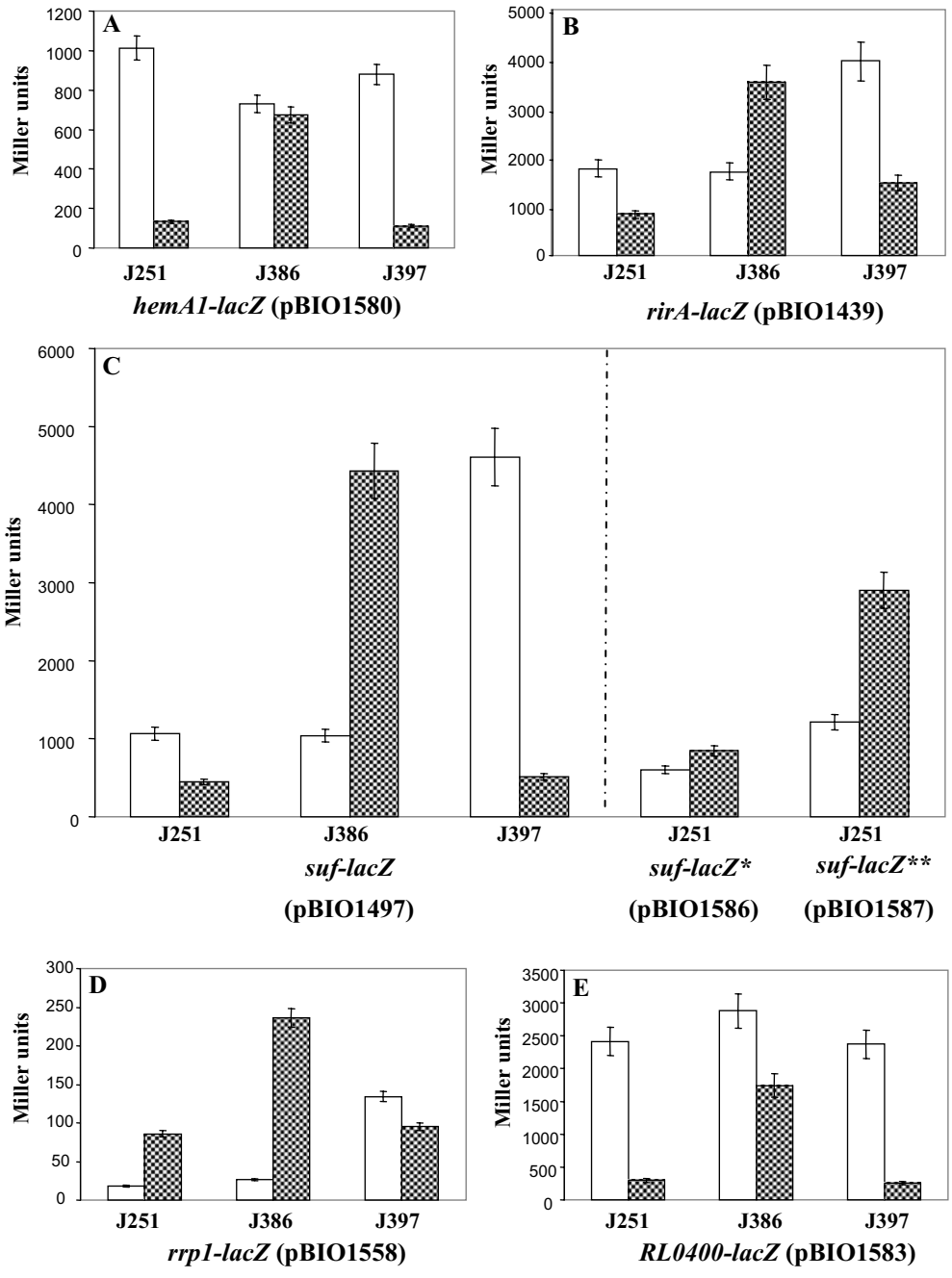

Fig. 2 Expression of transcriptional fusions in the *R. leguminosarum* genes related to iron metabolism. Strains of *R. leguminosarum* wild type (J251), IrrA$^-$ (J386) and RirA$^-$ (J397), each containing the *-lacZ* fusion plasmid shown were grown in minimal medium containing (*white bars*) or lacking (*hatched bars*) $FeCl_3$. In addition, derivatives of J251 wild type containing the mutated *suf* ICE motifs in pBIO1586 and pBIO1587 (shown as *suf-lacZ* fusions with * and ** respectively) were assayed (**c**). The strains were assayed in triplicate for *β*-galactosidase activity. Mean values, in Miller Units, are shown, together with the standard errors. The values shown are corrected for the background *β*-galactosidase activities of J251 (ca. 50 Units) and J386 (ca. 85 and 105 Units in + and −Fe respectively. These values take account of the fact that J386 has a Tn*lac* insertion that increases the levels of activity above the background in J251 (Wexler et al. 2003). Note that in J386, the fusion is chromosomal, but in Wexler et al. (2003) it is plasmid-borne, accounting for the higher levels in that report

synthesis of FeS clusters (Todd et al. 2005). In *E. coli*, the *suf* genes are repressed in +Fe media, under the control of the Fur regulator (Outten et al. 2004; Lee et al. 2004). In wild type *R. leguminosarum, sufS2BCDS1XA* was reported to be expressed at slightly lower levels in +Fe than in −Fe media, but in a RirA$^-$ mutant, its expression was much increased under +Fe conditions (Todd et al. 2005). This was interpreted to mean that, normally, *sufS2BCDS1XA* is repressed by RirA in +Fe conditions, consistent with the existence of an IRO motif near its promoter (see Fig. 3a). However, another (at that time, unknown) Fe-responsive regulator could act to increase its expression in high-Fe conditions, but its presence was only revealed if RirA was missing, as in the *rirA* mutant (Todd et al. 2005). To see if this unknown regulator was IrrA, the *suf-lacZ* fusion plasmid pBIO1497 was mobilized into J386, before assaying transconjugants for *β*-galactosidase. In this IrrA$^-$ mutant, the expression was much-enhanced after growth in −Fe, compared to the +Fe medium (Fig. 2c). In contrast, but as predicted from the earlier work, the reverse was true in the RirA$^-$ mutant containing pBIO1497 (Fig. 2c). Thus, *sufS2BCDS1XA* is indeed subject to regulation by both RirA and IrrA, such that in the absence of one of the regulators, the inhibitory effects of RirA in high-Fe and of IrrA in low-Fe media

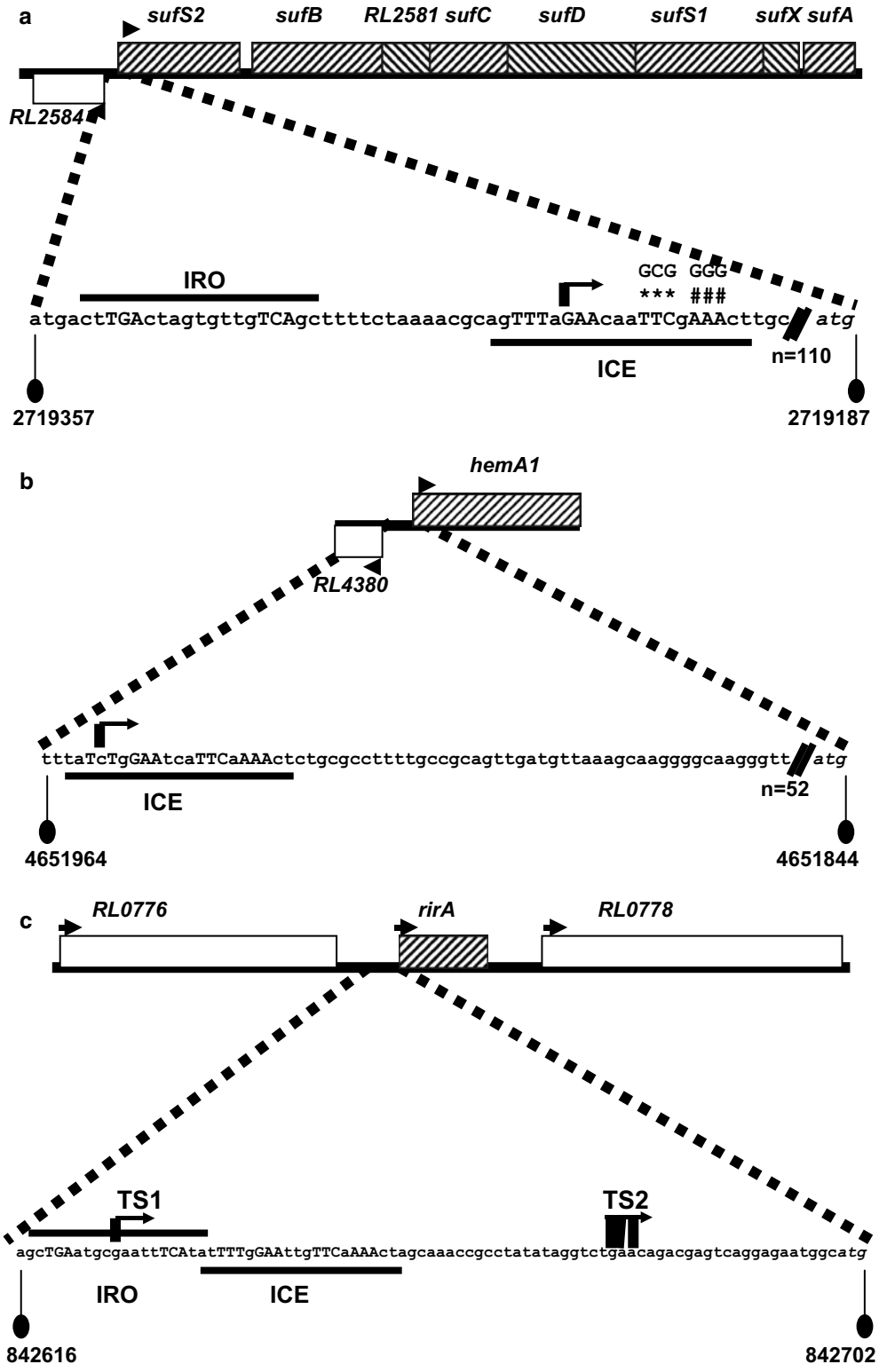

Fig. 3 Locations of IRO and/or ICE motifs preceding IrrA-regulated *suf, hemA1, rirA*, *rrp1* and *RL0400* genes. For each panel, the IrrA-regulated genes are shown as hatched boxes. Filled arrows indicate direction of transcription. The transcript starts, determined by primer extensions are shown as arrows above the DNA sequences. Note the two distinct transcript starts, TS1 and TS2 for *rirA* and the "stuttering" starts with *RL0400* and *rirA* TS2. Proposed ATG translational start sites are in italics. The numbers at the ends of the sequences are those in the genome of *R. leguminosarum* strain 3841 (ftp://www.ftp.sanger.ac.uk/pub/pathogens/rl/RL.pep). The ICE and IRO motifs are, respectively, under- and over-lined, the highly conserved diagnostic nucleotides being in capitals. In panel "a" the three altered bases in the muted *suf* ICE motif in pBIO1586 are shown as "*" and the additional three mutant bases in pBIO1587 as "#"; the mutated forms of the sequence are shown above these symbols

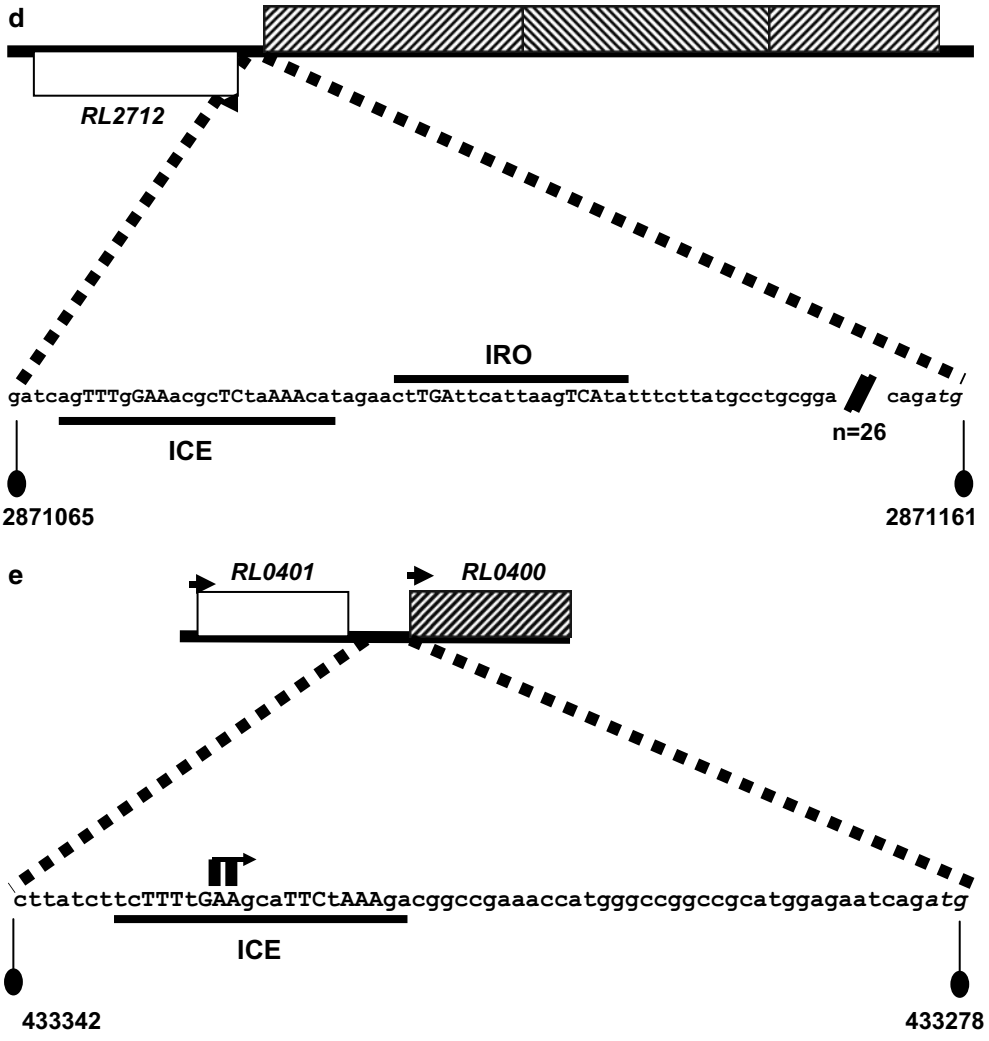

Fig. 3 (Contd.)

are revealed. Interestingly, in the wild type background, *sufS2BCDS1XA* expression was somewhat greater in +Fe than in −Fe medium. This differs from what we reported earlier, where the reverse was the case (Todd et al. 2005). Because of this discrepancy, we re-assayed the original wild type strain *R. leguminosarum* containing pBIO1497, which was used by Todd et al. (2005) and we reintroduced the exact same *suf-lacZ* reporter plasmid into J251 and into other wild type strains of *R. leguminosarum* in our collection. In all cases, expression of the fusion was greater in +Fe than in −Fe medium. Since the assays described by Todd et al. (2005) had been done repeatedly, it seems that unknown and, presumably, subtle, changes in the medium caused this difference. We discuss this unexplained observation below.

IrrA does not regulate previously identified genes involved in iron uptake

Having shown that IrrA regulates at least three sets of genes that are expressed at enhanced levels in +Fe medium, we investigated if it influenced transcription of genes that are known to be repressed by RirA in +Fe media. These included *vbs* and *fhu* genes that respectively specify the synthesis and the import of the siderophore vicibactin, and the *hmu* genes required for haem uptake (Carter et al. 2002; Stevens et al. 1999; Wexler et al. 2001; Yeoman et al. 2000). We therefore transferred into the IrrA$^-$ mutant a series of reporter plasmids in which the promoters of the *vbsC*, *fhuA* and *hmu* genes had been cloned 5′ of *lacZ* in pMP220. All displayed the same pattern of expression as in the wild type background, with much higher β-galactosidase levels in −Fe than in +Fe medium (data not shown). Thus, at least some Fe-regulated genes are negatively regulated by RirA in response to Fe but are unaffected by IrrA.

A conserved sequence that may be a target for IrrA recognition

Given that IrrA regulates *hemA1*, *rirA* and *sufS2BCDS1XA*, we inspected their promoter regions

for any conserved sequence features. We had already identified the two *rirA* transcriptional initiation sites, TS1 and TS2 (Todd et al. 2002) and the single TS for *sufS2BCDS1XA* (Todd et al. 2005). We identified the *hemA1* transcript start by isolating RNA from the wild type, and the IrrA$^-$ and RirA$^-$ mutants each grown in Fe-depleted and Fe-replete media, then performing primer extensions on these samples. There was a single transcript start, located 114 bps 5′ of *hemA1* (see Fig. 3b).

We identified a highly conserved sequence motif that spans the *hemA1* and *sufS2BCDS1XA* transcript starts and which is just 3′ of TS1 of *rirA* (Fig. 3c). This motif, AnTTTRGAAYnRTTCYAAAnT, has inverted repeat symmetry and resembles the iron control element (ICE) identified by Nienaber et al. (2001) as a likely Fe-responsive, *cis*-acting sequence for the *hmu* genes in *B. japonicum*, which can bind to Irr_{Bj} (Rudolph et al. 2006). This sequence has very little detectable similarity to canonical *fur* boxes or to the MRS box, to which the Fur-like, Mn^{2+}-responsive regulator Mur of *Rhizobium* binds (Supplementary Table 2). In the *sufS2BCDS1XA* and *rirA* operons, which are also subject to RirA-dependent repression in Fe-replete conditions, their IRO sequences (TGA-9 bp-TCA) lie upstream of their ICE motifs (Fig. 3a, c).

Site-directed mutagenesis reveals the importance of the ICE motif for IrrA-dependent regulation of the *sufS2BCDS1XA* operon

To confirm the importance of the ICE motif in IrrA-dependent gene regulation, two mutated forms of the *suf-lacZ* reporter plasmid pBIO1497 were made. In one of these, the highly conserved TTC in the ICE motif was mutated to GCG, to form plasmid pBIO1586 (see Fig. 3a). The other mutant plasmid (pBIO1587) had these same three mutations but the highly conserved AAA of the ICE motif was also mutated, to GGG (Fig. 3a). These two mutant plasmids were mobilized into wild type *R. leguminosarum* and the transconjugants were assayed for β-galactosidase after growth in +Fe and −Fe medium.

Replacement of 6 bps of the ICE motif, in pBIO1587, had a very significant effect on the expression of the *suf-lacZ* fusion, the levels of β-galactosidase being increased ~sevenfold in the −Fe medium compared to the wild type reporter pBIO1497 (Fig. 2c) Interestingly, in pBIO1586, in which only three of the conserved bases of the ICE motif were mutated, had intermediate expression, between those of the wild type and pBIO1587 (Fig. 2c). These observations confirm the importance of the ICE motif, 5′-AnTTTRGAAYnRTTCYAAAnT-3′, with its 12 particularly well-conserved nucleotides (underlined). The substitution of both the "TTC" and "AAA", at the 3′ end of the motif almost abolished IrrA-mediated repression, but if the latter was retained, some regulation remained. Thus, both these trimers are involved in the functioning of the ICE motif. It will be of interest to see if the same is true for the "TTT" and "GAA" at the 5′ end of the ICE motif, which are in inverse complementarity to the bases that were mutated here.

Identification of ICE sequences in the genome of *R. leguminosarum*

To identify other candidate members of the IrrA regulon, the recognition profile for the ICE motif was used to scan the genome of *R. leguminosarum* 3841. In addition to *hemA1, sufS2BCDS1XA* and *rirA*, five other *R. leguminosarum* transcriptional units have ICE sequences in their likely *cis*-acting regulatory regions, and, significantly, all of these have obvious links with iron metabolism (Table 3). For example, *RL0322*, or *irpA*, resembles an uncharacterized iron-responsive gene from *Synechococcus* sp. PCC 7942 that is required for Fe-limited growth (Durham et al. 2003), and *RL2703* is predicted to encode the fumarate hydratase FumA, a Krebs cycle enzyme containing a [4Fe–4S] cluster. Three other genes with ICE sequences in their regulatory regions were of interest for several reasons, as follows.

The *RL2713-RL2714-RL2715* operon likely encodes a ferri-siderophore ABC transporter similar to the iron-DtxR-regulated IRP6 transporter from *Corynebacterium diphtheriae* (Qian et al. 2002). Interestingly, the *RL2713* product corresponds to Rrp1, whose production was shown in a proteomic analysis to be enhanced in Fe-depleted conditions and to be regulated by RirA (Todd et al. 2005). As with the *sufS2BCDS1XA* and *rirA* promoter regions, the *rrp1* gene has both an ICE and IRO motif (Fig. 3d), suggesting that it might be regulated by both IrrA and by RirA (see below).

The product of *RL0263*, has an N-terminal domain with significant similarity to the bacterioferritin-like rubrerythrin (PFAM accession number PF02915), but which also, and most unusually, has a C-terminal domain in the DUF125 family (PF01988), which is predicted to be an inner membrane protein. Significantly, *R. leguminosarum RL0263* is an ortholog of the *blr7895* gene that Rudolph et al. (2006) found to have an ICE motif and to be expressed at higher levels in *B. japonicum* in Fe-replete conditions, most likely under the control of Irr.

Another *R. leguminosarum* gene with an ICE motif is *RL0400*, whose product is remarkably similar (~48% identity) throughout its length to a eukaryotic protein, known as Nfu1 in yeast and HIRIP5 (HIRA-interacting protein 5, the HirA protein being a transcriptional regulator of histone gene transcription) in mammals. HIRIP5 and Nfu1 are thought to act as scaffolds to transfer FeS clusters to different acceptor proteins (Lorain et al. 2001). Among prokaryotes, the only bacteria with proteins sharing extended homology to the *RL0400* gene product are in the α-proteobacteria. However, there is low-level homology between the

Table 3 Presence of ICE sequences in *R. leguminosarum* and in related bacterial species

Gene ID [a]	Operon [b]	Function [b]	ICE sequence [c]	Position [d]	Score [e]	ICE conservation in other species [f]
RL2583	*sufS2BCDS1XA*	FeS cluster synthesis	AGTTTAGAACaATTCgAAACT	−132	6.36	Sme, Mlo, Msp, Atu, Bme, Bqu, Bja, Rpa, Stm, Spo
RL0263	*RL0263*	Membrane-bound ferritin?	gaTTTAGAATtATTCTAAAaT	−35	6.06	Sme, Mlo, Msp, Atu, Bme, Bqu, Bja, Rpa, Rca, Rsp, Spo
RL0777	*rirA*	Fe-responsive regulator	AtTTTGGAATtGTTCaAAACT	−68	5.95	Sme, Mlo, Atu, Bme
RL4379	*hemA1*	haem synthesis	taTcTGGAATcATTCaAAACT	−113	5.36	Sme, Atu
RL0400	*RL0400*	FeS scaffold protein?	tcTTTtGAAgcATTCTAAAga	−43	5.30	Sme, Mlo, Msp, Atu, Ssp, Spo
RL0322	*irpA*	Fe-regulated protein (*Vibrio*)	ttcTTAaAATcATTCCAAACT	−205	5.24	Sme, Mlo, Atu
RL2703	*fumA*	Fumarate hydratase class I	gaTTTAGAcCgGTTCCAAtCT	−55	4.91	Bja, Rpa
RL2713	*rrp1 (irp6)*	Ferrisiderophore ABC transporter?	AGTTTGGAAacGcTCTAAAac	−93	4.79	Sme, Atu
Consensus			agTTTrGAAynrTTCyAAAct			

[a]Gene ID numbers are as in the *R. leguminosarum* strain 3841 genome project (http://www.sanger.ac.uk/Projects/R_leguminosarum/)
[b]Gene nomenclature and likely or proven function, if known
[c]Uppercase nucleotides correspond to those that match the ICE consensus (last line), where 'r' stands for either A or G; 'y' stands for T or C
[d]The positions of ICE sites are given relative to annotated translation starts
[e]The ICE site scores are computed using positional nucleotide weight matrix, as described in Materials and methods
[f]Only genomes with conserved ICE sites (score > 4.75) 5′ of the corresponding transcriptional unit in *R. leguminosarum* are shown: *Sinorhizobium meliloti* (Sme), *Mesorhizobium loti* (Mlo), *Mesorhizobium sp.* BNC1 (Msp), *Agrobacterium tumefaciens* (Atu), *Brucella melintensis* (Bme), *Bartonella quintana* (Bqu), *Bradyrhizobium japonicum* (Bja), *Rhodopseudomonas palustris* (Rpa), *Rhodobacter capsulatus* (Rca), *Rhodobacter sphaeroides* (Rsp) *Silicibacter pomeroyi* (Spo), and *Silicibacter* sp. TM1040 (Stm). For additional details on the candidate ICE sequences in those genomes see Supplementary Table 1

C-terminal region of the *RL0400* gene product and parts of NifU, a scaffold protein that delivers FeS clusters to nitrogenase (Agar et al. 2000).

Interestingly, we noted that these newly identified ICE motifs also occur 5′ of orthologous genes in several other members of the Rhizobiales and Rhodobacteriales, consistent with the presence of *irr* genes in these lineages (see Table 3 and Discussion).

Confirmation that *RL0400* and *rrp1* are regulated by IrrA

Having identified several new transcriptional units that are predicted to be regulated by IrrA, we directly examined the behavior of two of them, *RL0400* and the *rrp1* operon. We cloned the promoter regions of *rrp1* and *RL0400* into pMP220 to form pBIO1558 and pBIO1553 respectively, then mobilized both plasmids into the wild type and into the IrrA$^-$ and RirA$^-$ mutants of *R. leguminosarum*. The transconjugants were grown in +Fe and −Fe media, before assaying *β*-galactosidase activities.

The *β*-galactosidase assays with the *rrp1-lacZ* fusion pBIO1558 showed that it was regulated by Fe, the activity being substantially less in the +Fe media when the fusion was in the wild type strain (Fig. 2d). Interestingly, the expression of *rrp1* was much enhanced in the IrrA$^-$ mutant, but only in −Fe media, showing that IrrA could repress *rrp1* expression in such conditions. Also, and as predicted from the proteomic study (Todd et al. 2005), Fe-dependent repression in +Fe media was lost in the RirA mutant J397 (Fig. 2d). Thus, like *rirA* and *sufS2BCDS1XA*, the *rrp1* gene is regulated by both IrrA and RirA, consistent with the presence of IRO and ICE motifs in its likely regulatory region (Fig. 3d).

In contrast, *RL0400* is regulated by IrrA but not RirA. Thus, in both the wild type and RirA$^-$ mutant backgrounds, *RL0400-lacZ* was expressed at higher levels in +Fe than in −Fe media, but in the IrrA$^-$ mutant, expression in −Fe media was only slightly reduced, compared to when the cells were grown in +Fe medium (Fig. 2e).

To locate the *RL0400* ICE motif relative to its transcriptional initiation site, primer extensions were done. These showed higher levels of expression in the IrrA$^-$ mutant than in the wild type, consistent with the *lacZ* fusion data (Fig. 4). The *RL0400* transcriptional start site was in the same position in the wild type and in the IrrA$^-$ mutant and was located within the ICE motif that had been predicted above (Fig. 3e). The DNA in the vicinity of the *RL0400* transcript start had no detectable IRO motif, consistent with the fact that its expression was unaffected by the *rirA* genotype.

Discussion

The results presented here, together with previous studies on Fe-responsive gene regulation in *R. leguminosarum*, confirm and extend our previous conclusions

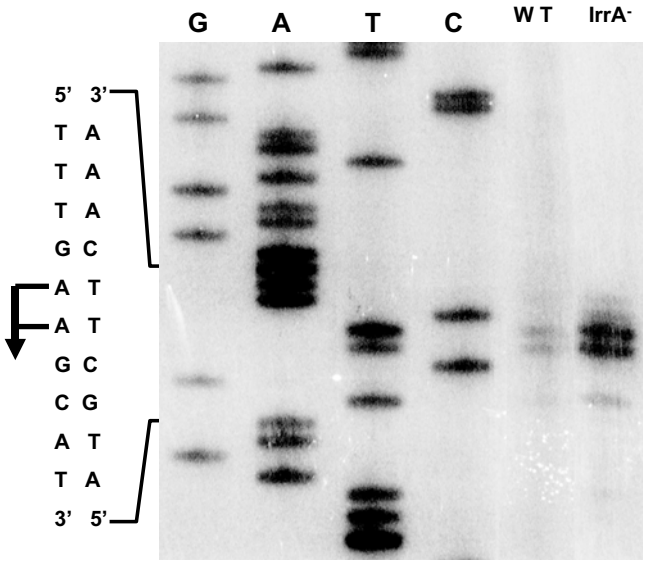

Fig. 4 Location of *RL0400* transcriptional start by primer extension. Using RNA obtained from wild type and IrrA^{-} mutant *R. leguminosarum* grown in Fe-depleted media and the primer *RL0400*PE (Table 2) the 5′ ends of the *RL0400* transcripts are shown in relation to the sequence of the corresponding region, which was determined using the same primer and the cloned *RL0400* promoter DNA as template

that the regulatory response to this metal is very different and more complex than in *E. coli* and other bacteria whose primary Fe-responsive regulator is Fur. It is clear that *R. leguminosarum* has two wide-ranging regulators, IrrA and RirA, which probably act, respectively, on the ICE and IRO motifs in the operator regions of their own sets of target genes. Intriguingly, they appear to respond in opposite senses to external Fe concentrations, IrrA being a repressor in Fe-deficient media and RirA in Fe-replete conditions. Since at least some genes are regulated by both IrrA and RirA, there are at least three sets of Fe-responsive genes, some being regulated by IrrA, some by RirA and some or by both.

Those genes that are most obviously involved in the acquisition of Fe are, not surprisingly, expressed at higher levels in −Fe media, this being mediated by the RirA protein, most likely acting on the IRO motifs of (e.g.) the *fhu, vbs* and *hmu* genes (Todd et al. 2002, 2005). As shown here, such genes are unaffected by the IrrA regulator.

The genes in the second class, of which we have identified two examples, *hemA1* and *RL0400*, are transcribed at higher levels in +Fe media, this regulation involving IrrA but not RirA. The *hemA* of *B. japonicum* is also expressed at higher levels in response to Fe availability, but this is mediated by Fur, not by RirA (Hamza et al. 2000). Interestingly, transcription of the single *hemA* gene of *B. japonicum* is also affected by O_2 availability, this being mediated by the FixK regulator (Hamza et al. 2000; Page et al. 1994; Page and Guerinot 1995). It is unclear why *R. leguminosarum* has two *hemA* genes, one of which (*hemA1*) is regulated by Fe via the IrrA regulator and the other, (*hemA2*), which responds to O_2 availability, being virtually quiescent in aerobic conditions. It will be of interest to know which, if either, of the *R. leguminosarum hemA* genes is important in supplying haem in nodules.

The product of the other gene, *RL0400*, which is regulated by IrrA but not RirA and whose expression is enhanced in +Fe media, is a homologue of the likely FeS-transfer protein HIRIP5, as well as of proteins in other α-proteobacteria. The exact function of the *RL0400* gene product is unknown, but presumably involves a process that occurs more actively at higher levels in Fe-replete conditions. We did not show directly that *RL0263*, a third gene with an ICE motif near its likely promoter, is regulated by IrrA. However, it seems likely that this is the case, given that Irr_{Bj} can bind to the ICE motif of the corresponding gene in *B. japonicum* (Rudolph et al. 2006). As with that of *RL0400*, the function of the *RL0263* gene product is unknown, but its very unusual domain structure suggests that it might be an inner membrane protein that can bind to Fe and hence may afford resistance to the metal; if so, it would be an advantage to be expressed in Fe-replete media. Homologues of *RL0263* occur in eleven other analyzed bacteria from the Rhizobiales and Rhodobacteriales orders and in all cases, they have ICE sequences in their likely regulatory regions (Table 3, Supplementary Table 1), suggesting that the *RL0263* gene product has an important role in the Fe metabolism in these bacteria.

In terms of their regulation, perhaps the most interesting sets of genes are those that are regulated by both IrrA and RirA. We identified three of these, *sufS2BCDS1XA*, *rirA* and *rrp1*, all of which contained both an ICE and an IRO motif in their operator regions. In *R. leguminosarum*, it seems that under our growth conditions RirA-mediated repression exerts a greater influence than IrrA in the regulation of *rrp1*, since in J251 the *rrp1-lacZ* fusion was expressed at higher levels in −Fe than in +Fe medium. In contrast, for *sufS2BCDS1XA* and *rirA*, the reverse was true, suggesting that the repressive effect of IrrA in −Fe medium was more important than the RirA-mediated repression in +Fe medium. We cannot explain why we now find that the *sufS2BCDS1XA* operon is expressed at higher levels in +Fe medium, whereas previously the reverse was true (Todd et al. 2005). We can only suggest that there may be some difference in the media when the two sets of experiments were done, and that this caused a switch from RirA being the dominant regulator to a situation in which the effects of IrrA are more pronounced. Perhaps only minor differences may tip the balance between an overall appearance of induction or repression in response to extracellular Fe concentrations.

We had shown (Todd et al. 2002) that *irrA* expression is regulated by RirA in +Fe media; the fact that IrrA also regulates *rirA* transcription reveals an even more intimate link between these two regulators, over and above the fact that they can act on the same genes.

R. leguminosarum and *B. japonicum* each have a second *irr* gene, of unknown function(s) which we term *irrB*; in contrast, the genome of S. *meliloti* has only one *irr* gene; but the significance of this is not clear. Despite the wide range of genes regulated by *R. leguminosarum irrA* and/or *rirA*, it is surprising that mutations in neither gene affect symbiotic N_2 fixation on peas (Todd et al. 2002; Wexler et al. 2003). It remains to be seen if this is because a different regulon comes into action within the bacteroids.

A microarray study lists ~300 *S. meliloti* genes whose expression was affected (either greater or lower) by Fe availability and 200 whose transcription differed in the wild type compared to that in a $RirA^-$ mutant (Chao et al. 2005). Not surprisingly, there was some overlap between the two sets of data and it remains to be seen how many of the genes whose expression is affected by Fe but not by RirA are regulated by IrrA. For reasons that are not clear, some of the genes that are regulated by RirA in *R. leguminosarum* (e.g. *tonB*) were not detected by Chao et al. (2005) as being differentially expressed nor were the IrrA-regulated genes *RL0263* or *RL0400* even though *S. meliloti* has orthologues of all these genes, and have IRO or ICE sequences as appropriate.

The Irr transcriptional regulator was first identified in *B. japonicum* as a regulator of *hemB*. Ironically, as pointed out by Rudolph et al. (2006), this *hemB* has no ICE motif, so the first gene shown to be regulated by Irr may be controlled by a different mechanism that does not involve direct binding of Irr_{Bj} to an ICE motif. In *R. leguminosarum*, IrrA is not involved in the control of *hemB*, but, instead, it regulates *hemA1*. In +Fe conditions, Irr_{Bj} is highly unstable, and so cannot exert its repressive abilities. This post-translational instability in Fe-replete media is mediated by haem availability, through a complex interaction whereby haem is delivered by ferrochelatase to an HRM and to at least one more site in the Irr_{Bj} protein. This provides a nice system of feedback control, linked to the flux through the haem biosynthetic pathway (Qi and O'Brian 2002; Yang et al. 2005). It remains to be seen if the *R. leguminosarum* IrrA is unstable in response to haem, particularly since it lacks the high affinity HRM, though it does have the "HHH" motif that is involved in the interaction with haem in *B. japonicum* (Wexler et al. 2003; Yang et al. 2005). In fact, the only Irr proteins to have a high affinity HRM are those of *B. japonicum* and the very closely related *Rhodopseudomonas palustris* (Fig. 1). However, Irr of *Brucella* can bind to haem in vitro, even though it, too, lacks the high affinity HRM (Martinez et al. 2005). As in *Rhizobium*, Irr^- mutants of *Brucella* accumulate protoporphyrin, showing that there is a link with haem biosynthesis even when the Irr has no high affinity HRM motif.

As stated, ICE motifs precede several Fe-responsive genes in *B. japonicum*. Interestingly, these motifs occur in the *cis*-acting regulatory regions of some genes whose expression was enhanced in −Fe compared to +Fe conditions and in others where the reverse was true. The former group included the *hmu* genes involved in haem uptake. In *R. leguminosarum*, the *hmu* genes are repressed by RirA, but not by IrrA. In the Fe-repressed genes of *B. japonicum*, the ICE sequences are located some distance upstream of the promoters, but in those genes whose expression is enhanced in +Fe media, the ICE motif overlaps their promoters, just as in *R. leguminosarum*. Interestingly, Irr_{Bj} could bind in vitro to these promoters but not to those, (e.g. *hmu*) whose ICE motifs were upstream and appeared to act positively on gene expression (Rudolph et al. 2006). Similar work, using purified protein from *R. leguminosarum* will show if its IrrA binds to all of the ICE motifs that we have identified.

In *E. coli, B. subtilis* and *P. aeruginosa* (and several other genera that have been studied less intensively), in which Fur is the global Fe-responsive regulator, the bacteria monitor iron levels directly, by a relatively simple process in which Fe^{2+} complexes with Fur, causing it to bind its cognate *fur* boxes and to repress transcription (Andrews et al. 2003). In *E. coli* even for those genes in which Fur appears to be an activator, the underlying mechanism is the same; Fur-Fe^{2+} represses *ryhB* expression, alleviating the inhibitory effects of RyhB sRNA on its various targets (Gottesman 2004). However, there is at least one case in which Fur can act directly as a positive regulator; Fur of *Neisseria meningitides* can activate or repress various genes required for virulence depending on its binding site, relative to the start of transcription (Delany et al. 2004).

Even taking these non-conventional behavior patterns of "classical" Fur into account, the work presented here on *R. leguminosarum*, together with our earlier work on this species and the observations of others on other rhizobia, prompt us to put forward a novel model for Fe-responsive gene regulation in these bacteria (see Fig. 5). We suggest that *R. leguminosarum* does not sense Fe availability, per se, but does so secondarily, through at least two, wide-ranging, transcriptional regulators, RirA and IrrA, which act in opposing ways. In this model, we propose that IrrA responds, directly or indirectly, to the intracellular haem concentration and that RirA senses the availability (and/or particular chemical form) of FeS clusters within the cell. It has been experimentally verified, at least for *B. japonicum* and *Brucella* (see above) that Irr interacts with haem and it seems likely that RirA is an FeS protein since it is the same Rrf2 superfamily as the ratified FeS protein IscR and preliminary evidence shows that purified *R. leguminosarum* RirA protein binds to FeS clusters in vitro (JDT, J. Crack and N. le Brun, unpublished observations). So, RirA may act as a repressor in response to the availability of FeS clusters, which, as with haem for Irr, may be a secondary, not a primary outcome of extraneous Fe availability.

Importantly, all five genera (*Agrobacterium, Bartonella, Brucella, Mesorhizobium* and *Sinorhizobium*) that are closely related to *R. leguminosarum*, as well as

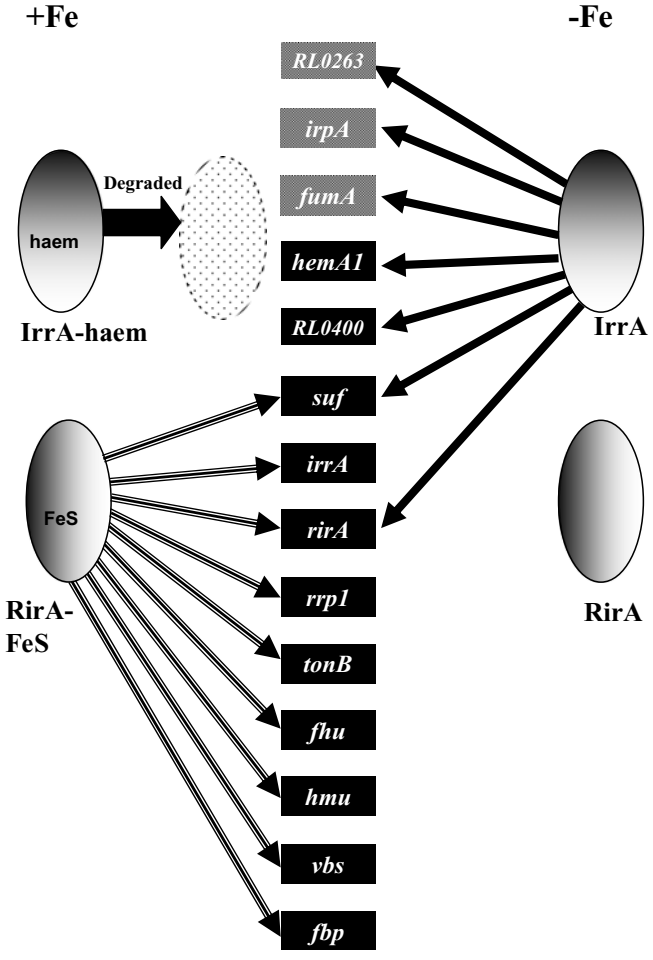

Fig. 5 Possible model for Fe-dependent gene regulation in *R. leguminosarum*. In Fe-replete conditions (*on the left*), RirA, bound to FeS clusters, represses transcription of the genes as indicated by striped arrows, whereas IrrA fails to function, due to haem-dependent instability. In Fe-replete media (*on the right*), RirA lacks its FeS cluster and does not repress, but IrrA represses its target genes (intact *arrows*). Those genes (*RL0263, irpA* and *fumA*) whose levels of expression were not assayed experimentally have a stippled background)

Rhodobacter and *Silicibacter*, not only have close homologues of RirA and of IrrA, but they have ICE or IRO motifs in the likely promoter regions of their *sufS2BCDS1XA*, *hemA1, RL0400, RL0263, rrp1* and *rirA* genes (Table 3; Table 1 in supplementary material). It is therefore likely that these bacteria, too, have a very different way of maintaining their intracellular iron homeostasis from that in other model, bacteria. Further work on the biochemistry and the physiology of iron metabolism of these and other, less well-studied bacteria may reveal still more novel features that relate to this important part of bacterial metabolism and gene regulation.

Acknowledgements Our thanks go to Nick le Brun and Jason Crack for useful discussions and to the BBSRC for funding. D.R. was supported by the Russian Fund of Basic Research (04-04-49361) and the Russian Academy of Sciences (Program "Molecular and Cellular Biology"). We are grateful to Gesine Rudolph and Hans-Martin Fischer for useful discussions.

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