Evidence that the *Rhizobium* regulatory protein RirA binds to *cis*-acting iron-responsive operators (IROs) at promoters of some Fe-regulated genes

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Mutations in *rirA* of *Rhizobium* have been shown to deregulate expression of several genes that are normally repressed by iron. A conserved sequence, the iron-responsive operator (IRO), was identified near promoters of *vbsC* (involved in the synthesis of the siderophore vicibactin), *rpoI* (specifies an ECF σ factor needed for vicibactin synthesis) and the two *fhuA* genes (encoding vicibactin receptor). Removal of these IRO sequences abolished Fe-responsive repression. Most of these genes were constitutively expressed in the heterologous host, *Paracoccus denitrificans*, but introduction of the cloned *rirA* gene repressed expression of these *Rhizobium* genes in this heterologous host if the corresponding IRO sequences were also intact. These observations are the first to examine the mechanisms of RirA, which has no sequence similarity to well-known iron-responsive regulators such as Fur or DtxR. They provide strong circumstantial evidence that RirA is a transcriptional regulator that binds to *cis*-acting regulatory sequences near the promoters of at least some of the genes whose expression it controls in response to Fe availability.

INTRODUCTION

Iron is needed as a component of many proteins. Although abundant on Earth, Fe acquisition presents difficulty because of its extremely low solubility in most biotic conditions, and microbes use many strategies to sequester it from their environments. Although essential, intracellular Fe is also potentially toxic, since it catalyses the formation of harmful radicals. Therefore the genes involved in Fe uptake, storage and utilization are tightly regulated in bacteria (see Andrews *et al.*, 2003).

In Gram-negative bacteria, the most widely used (and most studied) Fe-responsive gene regulator is Fur (ferric uptake regulator). In model γ -Proteobacteria (e.g. *Escherichia coli* or *Pseudomonas*), in the presence of Fe²⁺, this dimeric protein (Kolade *et al.*, 2002; Pohl *et al.*, 2003) binds to sequences, known as *fur* boxes, and prevents transcription of the target promoters. Fur[−] mutants in *E. coli* also have reduced expression of several transcripts (McHugh *et al.*, 2003). In some cases, this is via Fur-mediated repression of a small RNA molecule, RyhB, which then represses transcription of genes whose expression appeared to be induced by Fur (Masse & Gottesman, 2002).

Other Fe-responsive regulators include those of the 'Dtx' class, which are important in Gram-positive bacteria and cyanobacteria (see Andrews *et al.*, 2003). Although differing in their primary sequences, the structures of the Dtx and the Fur families share broad structural similarities (see Pohl *et al.*, 2003).

A third class of wide-ranging, Fe-responsive transcriptional regulators, termed RirA, was recently described for *Rhizobium leguminosarum*, the symbiotic N₂-fixing α -proteobacterium that induces root nodules on peas, beans and clover (Todd *et al.*, 2002), a process that is very demanding for iron (see Johnston *et al.*, 2001). Mutations in *rirA* cause deregulated expression of several genes whose transcription is normally repressed in cells grown in Fe-replete medium. These include the *vbs* and *fhu* genes, which are involved, respectively, in the synthesis and uptake of the siderophore vicibactin (VB) (Carter *et al.*, 2002; Stevens *et al.*, 1999; Yeoman *et al.*, 2000); *hmuPSTUV*, which specifies a haem ABC transporter (Wexler *et al.*, 2001); *tonB* (Wexler *et al.*, 2001); and *rpoI*, which encodes an ECF σ factor that specifically recognizes promoters of the *vbsADL* and *vbsGSO* VB biosynthetic genes (Yeoman *et al.*, 1999, 2003). Transcription of *rirA* itself is enhanced in Fe-replete conditions and is auto-regulated (Todd *et al.*, 2002).

There is no detectable sequence similarity between RirA and members of the Fur or Dtx families, suggesting that RirA represents a novel class of Fe-responsive regulator.

Abbreviations: IRO, iron-responsive operator; SDM, site-directed mutagenesis; VB, vicibactin.

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There are close homologues (>70 % amino acid identity) of RirA, but these are confined (so far) to *Sinorhizobium*, *Mesorhizobium*, *Agrobacterium* and the animal pathogen *Brucella*, all of which are near relatives of *Rhizobium*. However, RirA has ~30 % amino acid identity to the large (181 members) ‘Rrf2’ family, which occurs in all eubacteria, with many genera (including *Rhizobium*) having more than one paralogue. Rrf2 itself regulates cytochrome biosynthesis in *Desulfovibrio* (Keon *et al.*, 1997). The only other member to have been studied in detail, IscR of *E. coli*, regulates the adjacent *iscRSUA* operon, which is involved in the synthesis of FeS clusters. IscR is itself an FeS-cluster protein, repressing *iscRSUA* transcription, but is only fully effective if charged with this co-factor (Schwartz *et al.*, 2001). IscR does not repress the *iscRSUA* operon in Fe-depleted or oxygen-stressed *E. coli* cells, indicating that this regulator responds to both of these environmental signals (Outten *et al.*, 2004).

It is possible that a subset of the Rrf2 superfamily evolved into a specialized Fe-sensing regulator in *Rhizobium* and its very close relatives, with RirA mediating many functions that in *E. coli* are undertaken by Fur. Consistent with this, *Rhizobium* has a gene whose product resembles a ‘classical’ Fur, but mutations in the *fur*-like gene do not affect Fe-responsive regulation of the *fhu*, *vbs*, *rpoI*, *hmu* and *tonB* genes alluded to above (Wexler *et al.*, 2003). Indeed, this Fur-like protein is actually a manganese-sensing regulator, termed ‘Mur’, which represses the operon that specifies a Mn²⁺ ABC transporter, SitABCD, in Mn²⁺-replete cells (Diaz-Mireles *et al.*, 2004; Chao *et al.*, 2004).

Here we try to elucidate how RirA regulates Fe-responsive genes in *R. leguminosarum* and identify potential RirA-binding sites near promoters of some of its target genes.

METHODS

Strains, plasmids, media and genetic manipulations in vivo and in vitro. Strains and plasmids are described in Tables 1 and 2.

R. leguminosarum and *E. coli* were grown as described by Beringer (1974), and *Paracoccus denitrificans* as described by Carter *et al.* (2002). Techniques for conjugal gene transfer and assaying β-galactosidase in Fe-depleted media were as described by Wexler *et al.* (2001) and references therein.

Nucleic acid manipulations. Routine methods were used for cloning and plasmid isolations. Transcriptional fusions were made by cloning promoter fragments in the wide host-range reporter plasmid pMP220 (Spaink *et al.* 1987), in some cases having cloned the fragment initially in pUC18 (Messing *et al.*, 1983).

To map transcript starts (Sawers & Böck, 1989), cells were grown in Fe-replete or Fe-depleted media and total RNA was isolated. Using primers located within the structural, promoter-proximal gene, primer extensions were done, together with manual sequencing of the corresponding cloned segment of DNA to identify transcription initiation sites. The sequences of the primers used for these experiments were as follows: *vbsC* (5'-GATCGGCAGAGCTCCGCCTTG-3'); *fhuA1* (5'-CTTGTGAACACACGCAACTTTCCC-3'); *fhuA2* (5'-GGCTATCTCTATAAATTCGCG-3'); *fhuC* (5'-CGGCGACAA-AAGTTTCAGCCGTC-3').

Site-directed mutagenesis (SDM). The 10 bp deletion in IRO^{Ri} was generated using the ExSite kit (Stratagene) and primers that hybridized on either side of the designated deletion. The template DNA was plasmid pBIO1327, which contains a 400 bp DNA fragment spanning the *rpoI-vbsC* intergenic space, cloned in pUC18. Mutant plasmids were verified by DNA sequence analysis and the mutated insert was recloned into the wide host-range transcriptional fusion vector pMP220 in the appropriate orientation. Site-directed substitution mutations were generated using the QuikChange XL kit (Stratagene) to mutate the wild-type plasmid pBIO1328, which contains the same insert as in pBIO1327, but cloned in pMP220, forming a *rpoI-lacZ* fusion in pMP220. Mutagenic primers, 25 bp in length (synthesized by MWG-Biotech) and containing a single base pair substitution compared to wild-type (Table 2), were used in a PCR-based mutagenic reaction, and the products were transformed into *E. coli* DH10B-T1^R (Invitrogen), selecting tetracycline resistance. Mutations were verified by DNA sequencing. To mutate the IRO^{Vc}, the *vbsC-lacZ* fusion plasmid pBIO1306, which contains 780 bp, spanning the *rpoI-vbsC* intergenic space, cloned in pMP220 was used as a template. Using a QuikChange XL kit (Stratagene), 8 bp of IRO^{Vc} were removed and replaced with a *NotI* site (5'-GCGGCCGC-3') 16 bp downstream from the *vbsC* transcriptional start site. Mutations were verified by sequencing. The resultant

Table 1. Plasmids constructed and used in this study

Plasmid	Characteristics
pBIO1306	<i>vbsC-lacZ</i> transcriptional fusion, derived from pMP220 (tet ^r)
pBIO1327	<i>rpoI-vbsC</i> intergenic region cloned in pUC18
pBIO1328	pMP220-based <i>rpoI-lacZ</i> transcriptional fusion, derived from pBIO1327 (tet ^r)
pBIO1335	pMP220-based <i>rpoI-lacZ</i> fusion, deleted for IRO ^{Ri}
pBIO1354	pMP220-based <i>vbsC-lacZ</i> fusion, deleted for IRO ^{Ri}
pBIO1451	Intact <i>rirA</i> gene, plus its promoter, cloned in pOT2 (gent ^r)
pBIO1466	pMP220-based <i>vbsC-lacZ</i> fusion, lacks IRO ^{Vc}
pBIO1467	pMP220-based <i>rpoI-lacZ</i> fusion, lacks IRO ^{Vc}
pBIO1508	pMP220-based <i>fhuA1-lacZ</i> transcriptional fusion, contains IRO ^{Fa1}
pBIO1510	pMP220-based <i>fhuA1-lacZ</i> transcriptional fusion, lacks IRO ^{Fa1}
pBIO1512	pMP220-based <i>fhuA2-lacZ</i> transcriptional fusion, contains IRO ^{Fa2}
pBIO1514	pMP220-based <i>fhuA2-lacZ</i> transcriptional fusion, lacks IRO ^{Fa2}

Table 2. Effect of mutating individual nucleotides in IRO^{Ri} on the expression of *rpoI-lacZ* fusions

Plasmid pBIO1328 is an *rpoI-lacZ* fusion derived from pMP220 (see Methods). Derivatives of pBIO1328 with individual nucleotide substitutions (A→T, T→A, G→C or C→G) were made in the IRO^{Ri} sequence (see Methods); the altered bases are underlined and in bold type. Wild-type and mutant plasmids were mobilized to wild-type *R. leguminosarum* J251, and assayed in triplicate for β -galactosidase after growth in high- or low-level Fe media.

Plasmid	Nucleotide change	+Fe	-Fe
pBIO1328 (wild-type)	5'-TCGATTTAAACTTGA-3'	373 ± 59	908 ± 75
pBIO1474	<u>A</u> CGATTTAAACTTGA	458 ± 8	1428 ± 56
pBIO1484	T <u>G</u> GATTTAAACTTGA	376 ± 22	939 ± 32
pBIO1485	T <u>C</u> CATTTAAACTTGA	357 ± 7	811 ± 32
pBIO1486	TCG <u>T</u> TTTAAACTTGA	363 ± 15	1147 ± 52
pBIO1471	TCGA <u>A</u> TTAAACTTGA	412 ± 7	957 ± 12
pBIO1421	TCGAT <u>A</u> TAAACTTGA	392 ± 6	727 ± 25
pBIO1472	TCGATT <u>A</u> AAACTTGA	408 ± 3	856 ± 33
pBIO1420	TCGATT <u>T</u> AAACTTGA	450 ± 14	1245 ± 16
pBIO1473	TCGATT <u>T</u> ATACTTGA	658 ± 11	2248 ± 22
pBIO1427	TCGATT <u>T</u> AA <u>T</u> CTTGA	392 ± 42	1619 ± 159
pBIO1487	TCGATT <u>T</u> AAAGTTGA	322 ± 3	526 ± 2
pBIO1426	TCGATT <u>T</u> AAAC <u>A</u> TGA	326 ± 18	208 ± 4
pBIO1488	TCGATT <u>T</u> AAACT <u>A</u> GGA	299 ± 16	385 ± 11
pBIO1419	TCGATT <u>T</u> AAACTT <u>C</u> A	512 ± 7	331 ± 7
pBIO1489	TCGATT <u>T</u> AAACTT <u>G</u> T	376 ± 3	468 ± 12

mutant plasmid was termed pBIO1466. The mutant *rpoI-lacZ* fusion plasmid pBIO1467, which lacks IRO^{Vc}, was derived from pBIO1466, from which the insert was cloned into pMP220, but in the opposite orientation.

RESULTS

Location of IRO sequences relative to the transcript starts of *vbsC* and *rpoI*

In *R. leguminosarum*, RpoI is the σ factor responsible for transcribing the *vbsADL* and *vbsGSO* operons that specify enzymes involved in the synthesis of the siderophore VB (Yeoman *et al.*, 1999, 2003). Divergently transcribed from *rpoI* is *vbsC*, which specifies an enzyme that likely acetylates VB (Carter *et al.*, 2002). Expression of both *rpoI* and *vbsC* is reduced in wild-type, Fe-replete cells, but in *rirA* mutants this regulation is lost (Todd *et al.*, 2002). Transcription of *vbsC* and *rpoI* is normal in *rpoI* mutants; thus RpoI is not the σ factor for either of these genes (Carter *et al.*, 2002).

In the 163 bp intergenic region between *rpoI* and *vbsC* of *R. leguminosarum* strain J251, we noted two similar (11 of 17 bp identical) sequences, termed iron-responsive operators (IROs), with opposing orientation (see Fig. 1). Similar sequences occur near other Fe-regulated promoters (see below). The conserved sequence 5' of *rpoI*, which we term IRO^{Ri}, is centred 40 bp 5' of the previously identified (Yeoman *et al.*, 2003) transcription initiation site of *rpoI*. The other, termed IRO^{Vc}, is immediately 5' of the *rpoI* transcript start site (Fig. 1).

To locate IRO^{Vc} precisely, relative to the *vbsC* promoter, the *vbsC* transcription initiation site was identified by primer extension, using total RNA isolated from *R. leguminosarum* grown in Fe-replete and Fe-depleted media. A single *vbsC* transcript was found, whose intensity was, as expected, greater with mRNA obtained from cells grown in low-Fe medium (Fig. 2). The IRO^{Vc} sequence was centred around 17 bp 5' of this *vbsC* transcriptional start site, closer than is IRO^{Ri} relative to the *rpoI* transcript start. The 5' ends of the *rpoI* and *vbsC* transcripts are separated by 34 bp; thus there is overlap in the promoter regions of each of these two divergently transcribed genes (Fig. 1).

Effect of mutating the IRO sequences on the expression of *rpoI* and *vbsC*

Given their locations, relative to the transcription initiation sites of *vbsC* and *rpoI*, IRO^{Vc} and IRO^{Ri} might be *cis*-acting elements involved in Fe-responsive gene expression. To examine this, a series of mutations was made in which parts of these IRO sequences were individually mutated and the effects on expression of *vbsC* and *rpoI* determined. To do this, similar fragments spanning the intergenic region were cloned, in both orientations, into the wide host-range promoter-probe *lac* fusion plasmid pMP220 to form pBIO1328 (*rpoI-lacZ* fusion) and pBIO1306 (*vbsC-lacZ* fusion). From each of these, mutant derivatives were constructed in which 10 bp were removed from the 5' end of IRO^{Ri} to form pBIO1335 (*rpoI-lacZ*) and pBIO1354 (*vbsC-lacZ*), respectively. Similarly, 8 bp from the 5' end of IRO^{Vc} in pBIO1328 and pBIO1306 were replaced by a

Fig. 1. (a) Architecture of the promoter regions of the RirA-regulated genes. Representation of the *rpoI*–*vbsC* intergenic region. Thick arrows show locations and orientation of genes in the *fhuA2F*–*vbsC*–*rpoI* region. The sequence of the *vbsC*–*rpoI* intergenic space is shown below. Locations of IRO^{Vc} and IRO^{Ri} are shown under the brackets, with the nucleotides that were removed (for IRO^{Ri}) or replaced (for IRO^{Vc}) by SDM (see text) shown by white type on a black background. The *rpoI* and *vbsC* transcription initiation sites are indicated, as are the distances to the translational starts of these two genes. (b) Comparison of 17 bp IRO sequences in the *fhu* and *vbsC* promoter regions. The 17 bp IRO^{Ri} sequence is shown at the top. Nucleotides in the IRO motifs 5' of the *fhuA1*, *fhuA2* and *vbsC* promoters that are identical to those in IRO^{Ri} are underlined. Distances (bp) to the corresponding transcript start sites are shown.

NotI site to form pBIO1467 and pBIO1466, respectively (Fig. 3). Each plasmid was mobilized into wild-type *R. leguminosarum* strain J251 and transconjugants, grown in Fe-replete or Fe-depleted media, were assayed for β -galactosidase.

As expected, with the two wild-type fusions, expression of both *vbsC*–*lacZ* and *rpoI*–*lacZ* was greater in cells grown in Fe-depleted medium (Fig. 3). As noted previously (Carter *et al.*, 2002; Yeoman *et al.*, 1999), with *rpoI*–*lacZ* there is still significant β -galactosidase enzyme activity even in high-Fe medium, but *vbsC*–*lacZ* expression is strongly repressed by Fe. Strikingly, the mutations in IRO^{Ri} (in pBIO1335) and IRO^{Vc} (in pBIO1466) caused a large increase in *rpoI*–*lacZ* and *vbsC*–*lacZ* expression, respectively, and totally abolished Fe-dependent repression (Fig. 3). Indeed, with these mutant derivatives, expression of *rpoI*–*lacZ* and *vbsC*–*lacZ* was enhanced, rather than repressed, by growth in Fe-replete conditions. Although the most striking effects of removing IRO^{Ri} and IRO^{Vc} were on the expression of *rpoI*–*lacZ* and *vbsC*–*lacZ*, respectively, there

was a significant enhancement of *vbsC* expression (in plasmid pBIO1354) when IRO^{Ri} was removed, although Fe-responsive expression was retained. This 10-mer deletion would potentially relocate the *vbsC* transcript start site (see Fig. 1a), perhaps enhancing the initiation of *vbsC* transcription. In contrast, the 8-mer IRO^{Vc} mutation in the *rpoI*–*lacZ* fusion plasmid pBIO1467 reduces *rpoI* expression, leading to constitutive low-level transcription in both high-Fe and low-Fe media (Fig. 2). Since IRO^{Vc} is located just 5' of the *rpoI* transcription start site, this effect may be due to the loss of binding of a *trans*-acting protein near the *rpoI* promoter, or it may arise from the alteration of the –10 sequence at the *rpoI* promoter.

Having established its importance in the control of *rpoI* expression, we examined IRO^{Ri} in more detail. Mutant derivatives of pBIO1328, which contains the intact *rpoI* promoter and regulatory region, were constructed by site-directed mutagenesis (SDM) in which individual bases of IRO^{Ri} were substituted. The resulting mutant plasmids were each mobilized into wild-type *R. leguminosarum* and

Fig. 2. Transcription of *vbsC* is regulated in response to iron levels. The transcription initiation site of *vbsC* was determined by primer extension with total RNA (25 µg) isolated from *R. leguminosarum* after growth in media with high and low concentrations of Fe. The DNA sequence was determined with the same primer and with a cloned DNA fragment spanning the corresponding region of the *vbsC* gene. The 5' end of the transcript is indicated by the arrow and the precise location of the initiation site relative to the DNA sequence is shown.

the transconjugants assayed for β -galactosidase activity after growth in high-Fe and low-Fe media. As shown in Table 2, most of these substitutions had little or no effect on *rpoI-lacZ* expression in either medium, but those at the 3' end of the motif abolished Fe-responsive repression of the fusion. However, these mutations at the 3' end of the IRO^{Ri} also reduced *rpoI* expression, even in $-Fe$ medium, indicating they affect promoter activity and the response to Fe availability. This is consistent with their location near the -35 region relative to the *rpoI* transcriptional start. Two other adjacent mutations, in pBIO1473 and pBIO1427 (Table 2), elevate *rpoI-lacZ* expression, especially in low-Fe medium. Although upstream from the -35 region, these two mutations may enhance *rpoI* promoter activity, either directly through interactions with RNA polymerase or, perhaps, by indirect interaction with another, unknown, factor.

Cloned *rirA* confers Fe-responsive repression of *rpoI* and *vbsC* in *P. denitrificans*

Carter *et al.* (2002) showed that the cloned *vbsGSO* and *vbsADL* genes were not expressed in the α -proteobacterium *P. denitrificans* unless the cloned *R. leguminosarum* σ factor gene *rpoI* was also present in this heterologous host. Thus, the promoters of *vbsGSO* and *vbsADL* are not recognized by a native *P. denitrificans* σ factor, but *rpoI* itself is expressed

				β -Galactosidase activity (Miller units)	
<u>Plasmid</u>	IRO^{Ri} →	IRO^{Vc} ←	<i>rpoI-lacZ</i>	+Fe	-Fe
pBIO1328	Intact	Intact		393±59	908±75
pBIO1335	Deleted	Intact		4424±112	2964±79
pBIO1467	Intact	Mutated		328±9	226±35
	IRO^{Vc} →	IRO^{Ri} ←	<i>vbsC-lacZ</i>	+Fe	-Fe
pBIO1306	Intact	Intact		117±14	798±31
pBIO1466	Mutated	Intact		3415±255	1452±131
pBIO1354	Intact	Deleted		576±17	1363±65

Fig. 3. Effect of mutating IRO^{Ri} and IRO^{Vc} on expression of *rpoI-lacZ* and *vbsC-lacZ* fusions. The two diagrams indicate the organization of the oppositely oriented inserts in the two parental transcriptional *lacZ* fusion plasmids pBIO1328 (*rpoI-lacZ*) and pBIO1306 (*vbsC-lacZ*), in both of which IRO^{Ri} (vertical stripes) and IRO^{Vc} (horizontal stripes) are intact. Mutant plasmids, which were deleted for 10 bp in IRO^{Ri} (pBIO1335 and pBIO1354) or which had an 8 bp substitution in IRO^{Vc} (pBIO1467 and pBIO1466) were derived from pBIO1306 and pBIO1328. The wild-type parental fusions and each mutant plasmid were mobilized into wild-type *R. leguminosarum* and the transconjugants grown in high-Fe and low-Fe media before assaying β -galactosidase enzyme activities in triplicate. Results, in Miller Units, are shown for each plasmid, for both media.

Table 3. Expression of *rpoI* and *vbsC* genes in *P. denitrificans* in the presence and absence of the cloned *rirA* gene

Transconjugants of *P. denitrificans* containing various *rpoI-lacZ* and *vbsC-lacZ* fusion plasmids (see Fig. 3) were assayed for β -galactosidase enzyme activity after growth in high-Fe and low-Fe media. In some strains, the cloned *rirA* gene, in pBIO1451, was also present.

Plasmids present	β -Galactosidase activity (Miller units)	
	+ Fe	-Fe
pBIO1328 (<i>rpoI-lacZ</i>)	2233 $\pm$ 42	2177 $\pm$ 54
pBIO1328 + pBIO1451 (<i>rirA</i>)	199 $\pm$ 2	515 $\pm$ 13
pBIO1335 (<i>rpoI</i> IRO ^{RiA} - <i>lacZ</i>)	1496 $\pm$ 21	1561 $\pm$ 25
pBIO1335 + pBIO1451	3690 $\pm$ 121	3922 $\pm$ 228
pBIO1306 (<i>vbsC-lacZ</i>)	275 $\pm$ 8	291 $\pm$ 6
pBIO1306 + pBIO1451	57 $\pm$ 1	82 $\pm$ 2
pBIO1466 (<i>vbsC</i> IRO ^{VcA} - <i>lacZ</i>)	198 $\pm$ 4	179 $\pm$ 10
pBIO1466 + pBIO1451	256 $\pm$ 2	170 $\pm$ 4

in this bacterium. We therefore directly examined *rpoI* expression in *P. denitrificans* and, for comparison, the divergently transcribed *vbsC* gene. Plasmids pBIO1328 and pBIO1306 (*rpoI-lacZ* and *vbsC-lacZ* fusions, respectively), were each mobilized into *P. denitrificans*, and the transconjugants were grown in medium with high and low concentrations of Fe. Both fusions were expressed in this host, but their expression was unaffected by the Fe status of the medium (Table 3), showing that *P. denitrificans* cannot mediate Fe-responsive regulation of *rpoI* or *vbsC*.

This suggested a genetic approach to test if *RirA* bound to IRO sequences near the *vbsC* and *rpoI* promoters. First, by using a *rirA-lacZ* fusion plasmid, we showed that *rirA* itself was expressed from its own promoter in *P. denitrificans* (not shown). We then cloned *rirA* into the wide host-range vector pOT2 (Allaway *et al.*, 2001), to form pBIO1451. [Although pOT2 was designed primarily to be used as a promoter-probe plasmid (it has a promoterless *gfp* gene 3' of its polylinker), in pBIO1451 *rirA* gene is expressed from its own native promoter.] This plasmid was then transferred (selecting the gentamicin resistance, specified by pOT2) into *P. denitrificans* harbouring the *rpoI-lacZ* or *vbsC-lacZ* fusion plasmids (see above). Transconjugants were grown in high-Fe and low-Fe media before assaying β -galactosidase enzyme activity. As shown in Table 3, the introduction of the cloned *rirA* gene caused a large reduction in the expression of both fusions. With the *rpoI-lacZ* fusion plasmid pBIO1328, this *RirA*-dependent repression was more marked in the high-Fe than in the low-Fe medium. However, with the *vbsC-lacZ* fusion pBIO1306, the repressive effects of introducing *rirA* were very similar in both sets of media.

To show that an IRO box is needed for this repression, the mutant *rpoI-lacZ* fusion plasmid pBIO1335, which has a

deletion in IRO^{Ri}, was transferred to *P. denitrificans*. As with its intact parent, pBIO1328, there was constitutive, high-level expression of *rpoI-lacZ* in *P. denitrificans* harbouring pBIO1335. Significantly, introduction of the cloned *rirA* gene no longer conferred Fe-responsive repression to this mutated *rpoI-lacZ* fusion (Table 3). For reasons that are not clear, the presence of *rirA* further enhanced the level of expression from pBIO1335.

With pBIO1466, the mutated *vbsC-lacZ* fusion plasmid that lacks an intact IRO^{Vc}, a similar pattern was found. Whereas the expression of β -galactosidase from the parental plasmid pBIO1306 was repressed by introducing cloned *rirA* into *P. denitrificans*, when IRO^{Vc} was removed, the inhibitory effect of *RirA* in this heterologous host was abolished (Table 3).

Taken together, these experiments, in which *P. denitrificans* was used as a 'null' background, provide strong circumstantial evidence that *RirA* protein acts directly on the IRO motifs of *vbsC* and *rpoI*, resulting in a very significant drop in the levels of transcription of both genes. In the case of the *rpoI-lacZ* fusion, this repression is much greater in the high-Fe than in the low-Fe medium just as it is in *R. leguminosarum* itself. However, with the *vbsC* fusion, *RirA* is almost equally repressive in both media. The reasons for this difference are unknown, but may suggest the presence of other regulatory factors that are needed for the precise regulation of some, but not all, *RirA*-responsive promoters. It might also be, though, that the presence of extra copies of the cloned *rirA* can confer repression of *vbsC*, even in the absence of Fe.

Different arrangements of *fhuA* genes in different field isolates of *R. leguminosarum*

In *R. leguminosarum* field-isolated J251, we had shown that the uptake of VB is mediated by the Fhu transport proteins, the outer-membrane receptor being FhuA, the periplasmic binding protein FhuD, the inner-membrane transporter FhuB and the ATPase FhuC (Stevens *et al.*, 1999; Yeoman *et al.*, 2000). Analysis of the genome of the sequenced *R. leguminosarum* field isolate 3841 showed that it has two *fhuA* genes, with '*fhuA1*' being on the symbiotic plasmid, pRL10JI, ~145 kb from the *nod* genes (http://www.sanger.ac.uk/Projects/R_leguminosarum/) and the other copy, '*fhuA2*' being in the *fhuA2F-rpoI-vbs* gene cluster on a different native plasmid, pRL12JI. The finding of two apparently intact copies of *fhuA* in strain 3841 was surprising, since field isolate J251 had been shown to have one intact *fhuA*, in the same location relative to *rpoI* as that of *fhuA2* in field isolate 3841. In the locus occupied by *fhuA1* in strain 3841, field isolate J251 has a non-transcribed pseudogene, ψ *fhuA*, with many stop codons, which, like *fhuA1* of 3841, is orientated divergently from *fhuCDB*. Despite this, ψ *fhuA* is 81 % identical over 460 bp at its 3' end to *fhuA1* of strain 3841. Thus, J251 probably had an intact *fhuA1*, but its function was lost by accumulated mutations (Yeoman *et al.*, 2000). In strain 3841, both

fhuA1 and *fhuA2* are functional: when individually cloned, each can confer VB uptake ability to a *fhuA* mutant of strain J251 (results not shown).

IRO-like sequences and the promoters of *fhu* genes involved in VB uptake

The reason for describing the differences in the organization of *fhuA* genes in these two strains is that when we examined the *R. leguminosarum* strain 3841 genome for IRO-like sequences (based on IRO^{Ri}), the closest match (14 of 17 identical, Figs 1a and 4) was located 5' of *fhuA2*. A near-identical sequence was also seen in the corresponding region 5' of *fhuA2* of field isolate J251. We also examined the DNA 5' of the other two *fhu* operons, *fhuA1* and *fhuCDB*, in strain 3841 for IRO-like sequences. As seen in Figs 1(b) and 4, there was a region 88 bp 5' of *fhuA1* with limited similarity to IRO^{Ri}, this being most pronounced at the 3' end of the motif, around the -35 bp region. In contrast, we found no IRO-like motifs near the transcript start of the *fhuCDB* operon, which had been located by primer extension experiments, even though *fhuCDB* expression is regulated in response to Fe availability, this being mediated by *RirA* (Todd *et al.*, 2002; unpublished observations).

To establish the roles, if any, of the IRO-like sequences ('IRO^{Fa1}' and 'IRO^{Fa2}') 5' of *fhuA1* and *fhuA2*, respectively, we first located their transcription-initiation sites, by primer extensions with RNA obtained from *R. leguminosarum* grown in high-Fe and low-Fe medium and with primers located in *fhuA1* or *fhuA2*. Both genes had a single transcription-initiation site, whose intensity was greater in the Fe-depleted cells (not shown). Thus, the IRO^{Fa1} motif overlaps the *fhuA1* promoter region (see Fig. 4). However, IRO^{Fa2} was ~50 bp 5' of the *fhuA2F* transcript start, more distant than in the other IRO motifs for the other genes described above (see Figs 1b and 4). The promoter regions of the *fhuA1* and *fhuA2F* operons were studied in more detail.

***fhuA1*.** A transcriptional *fhuA1-lacZ* fusion plasmid, pBIO1508, which contains DNA extending 60 bp 5' of the *fhuA1* transcriptional start, exhibited normal Fe-responsive expression in wild-type *R. leguminosarum* (Fig. 4). In the *RirA* mutant J397 (Todd *et al.*, 2002), expression of this *fhuA1-lacZ* fusion was at a high level, even in high-Fe medium (data not shown), formally demonstrating that *RirA* mediates Fe-responsive regulation of *fhuA1*. When the slightly smaller fusion plasmid

Fig. 4. Expression of various *fhu-lacZ* fusions in *R. leguminosarum* and *P. denitrificans*. The DNA sequences in the regions upstream of the transcriptional start sites of *fhuA1* and *fhuA2F* are shown. Sequences of the corresponding IRO motifs are shown as underlined bases. Two wide host-range *fhuA1-lacZ* fusion plasmids, pBIO1508 and pBIO1510, were constructed by amplifying fragments of 338 and 318 bp, respectively, from strain J391 genomic DNA. The 3' ends of these plasmids are within *fhuA1*, and their respective 5' ends are shown as the open, vertical arrows. Two *fhuA2-lacZ* fusion plasmids, pBIO1512 and pBIO1514, containing 369 and 198 bp, respectively, were constructed in a similar way. All PCR products had *EcoRI* and *SphI* sites at their termini, to facilitate cloning into pUC18, and thence into the wide host-range vector pMP220. Transconjugants of wild-type *R. leguminosarum* (Ri) or *P. denitrificans* (Pd) containing each of these four fusions were grown in high-Fe (+Fe) or low-Fe medium (-Fe) and assayed for β -galactosidase. In addition, strains containing these *lac* fusion plasmids plus the cloned *rirA* gene (in pBIO1451) were also assayed as indicated. Activities are shown in Miller units, the mean of three independent measurements. NT, Not tested.

pBIO1510, which lacks IRO^{Fa1}, was mobilized to wild-type *R. leguminosarum*, the expression of β -galactosidase was essentially the same in +Fe and -Fe media (Fig. 4). Thus, removal of IRO^{Fa1} abolishes Fe-responsive regulation of *fhuA1*.

These two *fhuA1-lacZ* fusion plasmids were also mobilized into *P. denitrificans*. In this background, both of them expressed β -galactosidase constitutively. Thus, as with *rpoI* and *vbsC*, this heterologous species can transcribe the introduced *R. leguminosarum* gene but cannot effect Fe-responsive regulation of its expression. When pBIO1451 (*rirA* cloned in pOT2), was introduced into *P. denitrificans* that harboured pBIO1508, there was significant reduction in the level of β -galactosidase activity. This was more pronounced in the high-Fe than in the low-Fe medium, but in both regimes there was still a significant level of expression, showing that the repression was not as effective as that of the analogous experiments using the *rpoI-lacZ* fusion plasmid in *P. denitrificans* (see above). Importantly, though, the presence in *P. denitrificans* of the cloned *rirA* gene had no effect on the expression from pBIO1510, which lacks an intact IRO^{Fa1} (Fig. 4).

The IRO^{Fa1} motif is therefore likely to be a *cis*-acting regulatory element that interacts with RirA, despite its considerable sequence divergence compared to IRO^{Ri} and IRO^{Vc}.

***fhuA2*.** As mentioned above, the best match to IRO^{Ri} in the genome of *R. leguminosarum* is to a motif (IRO^{Fa2}) 5' of the *fhuA2F* promoter, but further upstream of the *fhuA2F* promoter than were the other IRO sequences relative to their promoters. Nevertheless, this IRO^{Fa2} motif appears to be important in the Fe-responsive regulation of *fhuA2F*, as seen by the behaviour of two *fhuA2-lacZ* fusion plasmids in wild-type *R. leguminosarum* grown in high-Fe or in low-Fe medium. One of these, pBIO1512, which contains the intact IRO^{Fa2} and extends 87 bp 5' of the *fhuA2* transcriptional start site, exhibited Fe-responsive repression of β -galactosidase in *R. leguminosarum* grown in Fe-replete medium (Fig. 4). However, the smaller plasmid pBIO1514, whose 5' end is within IRO^{Fa2}, shows no Fe-dependent regulation, its expression being essentially the same in the +Fe and -Fe media.

In contrast to other *lac* fusions described here, when pBIO1512 was transferred to *P. denitrificans*, its expression was affected by the Fe availability of the medium, the levels of β -galactosidase being approximately twofold lower in the high-Fe than in the low-Fe medium. With the smaller fusion plasmid pBIO1514, which lacks an intact IRO^{Fa2}, no such Fe-responsive regulation was seen. This suggests that the region 5' of *fhuA2* contains a sequence that is recognized by an (unknown) Fe-responsive regulator that is native to *P. denitrificans*. Introducing the cloned *rirA* into *P. denitrificans* harbouring the *fhuA2-lacZ* fusion pBIO1512 had little further effect, the ratio of expression

in high-Fe and low-Fe media being similar (~ 0.3) to that when the *P. denitrificans* did not contain *rirA*.

This difference in behaviour of the *fhuA2* regulatory region in the heterologous host was not due to the use in *P. denitrificans* of a promoter different to that used in *R. leguminosarum*. From primer extension experiments, using RNA from *P. denitrificans* harbouring the cloned *fhuA2* gene, it was found that the *fhuA2* transcriptional start was identical in the two bacteria, and that the levels of expression were significantly greater in cells that had been grown in Fe-depleted medium (not shown).

DISCUSSION

Todd *et al.* (2002) showed that mutations in *R. leguminosarum rirA* cause constitutive expression of several genes involved in iron uptake, which are normally repressed under Fe-replete conditions. However, it was not determined whether the RirA protein was a transcriptional regulator that recognizes motifs near the promoters of its target genes or not. The only other member of the Rrf2 family to be studied in detail is IscR, which binds at the operator of the *iscRSUA* operon (Schwartz *et al.*, 2001). It would be surprising, *a priori*, if RirA is not a DNA-binding regulator, which may also contain an [FeS] cofactor.

Here, a combination of deletion analyses and expression studies of various Fe-responsive *Rhizobium* genes in *Paracoccus* has provided strong circumstantial evidence that RirA likely does recognize IRO motifs. Although this work revealed some similarities in the ways in which the IROs identified here affect Fe-responsive gene expression, there are also some differences, pointing to differences in the exact way in which RirA mediates its regulatory effects on individual target genes. In what follows, it should be kept in mind that although we know that expression of *vbsC*, *rpoI* and the *fhu* genes does not involve the RpoI σ factor, it has not been established whether transcription of one or more of these genes is mediated by the housekeeping σ^{70} or by one (or more) of the other σ factors that *Rhizobium* possesses. It cannot be discounted, therefore, that the different responses of the genes examined here may be due to their being transcribed by different σ factors.

Here, we have used an operational definition of a consensus of a 17-mer as the IRO sequence. However, since in some cases (*rpoI* and *fhuA1*) the 3' end of the IRO motif overlaps the -35 region of the promoter, we have not formally distinguished bases that may be conserved because they constitute part of the RNA polymerase recognition sequence from those that may be specifically involved in interacting with RirA. Indeed, some nucleotides may be involved in both these functions.

The most detailed studies were done on *rpoI* and the divergently transcribed *vbsC*. Removal (in the case of *rpoI*) or replacement (for *vbsC*) led to their enhanced expression, especially in medium that was Fe-supplemented. Thus, the

importance of these sequences in Fe-responsive regulation is unequivocal. The locations of the IRO^{Ri} and IRO^{Vc} motifs relative to the transcription starts of the corresponding genes were different, but in both cases would be in accord with transcriptional repression of *rpoI* and *vbsC*. Further, SDM of individual bases in the 5' regions of the IRO^{Ri} motif had little or no effect on Fe-responsive regulation of *rpoI*. These sets of observations suggest that there may be considerable flexibility in the sequences required for the interaction with RirA. This was further borne out by the examination of the IRO-like sequences in the promoter regions of *fhuA1* and *fhuA2*. In the former, the IRO^{Fa1} motif overlaps the -35 region of the *fhuA1* transcript, just as does IRO^{Ri} relative to the *rpoI* promoter. However, the DNA sequences of IRO^{Fa1} and IRO^{Ri} are not strikingly similar, especially towards their 5' ends. The reverse is true for the IRO^{Fa2} motif, which strongly resembles the sequence of IRO^{Ri}, but is significantly further upstream of the *fhuA2F* promoter than are the other IROs from their corresponding transcription start sites. Such limited sequence similarity between factor-binding sites has been observed previously for other transcriptional regulators. For example, with OxyR from *E. coli*, the nucleotide contacts required for protein recognition are separated by 10 bp intervals (Toledano *et al.*, 1994) and different genes have different spacings between the OxyR-binding sites and the promoter.

All the fusions studied here were expressed in the heterologous α -proteobacterium *P. denitrificans*, but with one exception (*fhuA2F*) they were not subject to Fe-responsive repression in this background. The behaviour of most of the *lac* fusions in this study indicates that *P. denitrificans* does not have a functional equivalent of RirA. This is not unexpected, since close homologues of RirA exist only in the very near relatives of the rhizobia and are not found even in other α -proteobacteria.

However, the Fe-responsive repression of the *fhuA2F-lacZ* fusion indicates that, at least in some cases, the regions 5' of Fe-regulated *R. leguminosarum* genes can be recognized by regulators in this α -proteobacterium. It is becoming increasingly clear that bacterial genomes are remarkably mosaic in form, with large regions having likely been acquired by lateral gene transfer (Lawrence & Hendrickson, 2003). Possibly, therefore, *fhuA2F* was acquired from another bacterium relatively recently, and had retained some of the *cis*-acting regulatory sequences that were used in its previous host, in addition to having acquired the cognate IRO sequences appropriate for RirA-dependent regulation in *R. leguminosarum*. Thus, it might be potentially subject to one of two different mechanisms of Fe-responsive regulation, one appropriate for *Paracoccus* and the other for *Rhizobium*. We do not know what is/are the Fe-responsive regulator(s) in *Paracoccus*. Indeed, little or nothing is known of Fe-responsive gene regulation in any α -proteobacterium, even in such genetically well-characterized genera as *Caulobacter* and *Rhodobacter*.

However, the lack of Fe-responsive regulation of most of the RirA-regulated operons studied here in *P. denitrificans* presented an effective means of studying the effects of RirA on its target genes in a heterologous null background. There was a striking repression of *fhuA1-lacZ*, *vbsC-lacZ* and *rpoI-lacZ* fusion expression when the cloned *rirA* was also introduced into *P. denitrificans*, and this required the corresponding cognate IRO motif to be present. Although the introduced *rirA* gene may affect the expression of another regulator, native to *P. denitrificans*, which in turn regulates the fusion plasmid, we feel that this is inherently unlikely. It was noticeable that in *P. denitrificans* the cloned *rirA* gene markedly reduced expression of *rpoI*, *fhuC* and *fhuA1*, even in cells that had been grown in Fe-depleted medium. With *rpoI-lacZ* and, to a lesser extent, with *fhuA2-lacZ*, growth in high-Fe medium enhanced the repression, but this was not the case with the *vbsC-lacZ* fusion. Therefore, *R. leguminosarum* (but not *P. denitrificans*) may have some other factor that contributes to its ability to sense the availability of Fe, ensuring the appropriate level of Fe-dependent control in the native host.

This is the first report to examine the factors involved in RirA-mediated gene regulation in response to Fe availability. Although IRO motifs are clearly important to the ability of RirA to repress gene expression, the differences in the exact behaviour of the promoters that were studied here point to other unknown factors that influence the ability of RirA to affect the expression of specific transcriptional units. Indeed, one of the operons, *fhuCDB*, whose transcription quite clearly responds to Fe availability in an RirA-dependent way, does not even have a recognizable IRO motif near its promoter.

In addition to the genes involved in the synthesis and uptake of VB, Todd *et al.* (2002) showed that the Fe-dependent repression of other *R. leguminosarum* genes, namely haem-uptake (*hmu*) genes, ABC transporter (*fhp*) genes and *tonB*, also requires RirA. The transcriptional start sites of the *hmuPSTUV* and the *orf1-tonB* operons have been identified (Wexler *et al.*, 2001); in both cases, a sequence similar to the 3' end of an IRO motif was located at the ~ -35 position. More recently, proteomic studies have shown that >100 polypeptide gene products are differentially expressed in *rirA* mutant strains of *R. leguminosarum*, compared to the near-isogenic wild-type strains (J. D. Todd, unpublished observations). The transcript initiation sites for the corresponding genes have not been determined, but it does appear that at least some of these newly discovered RirA-responsive genes do not have a motif with similarity to the IRO motifs in their putative promoter regions.

Lynch *et al.* (2001) identified a more 'local' form of Fe-responsive gene control in *Sinorhizobium meliloti*. In this species, *rhrA* specifies a positive regulator that activates expression of the *rhb* genes, which are involved in the synthesis of the siderophore rhizobactin 1021. It will be of interest to see if there is any interplay between the

wide-ranging regulator RirA and the more specialised RhrA of *S. meliloti*.

We already know that RirA participates in one regulatory cascade, repressing the transcription of *rpoI*, whose σ factor product is, in turn, involved in the transcription of the *vbsGSO* and *vbsADL* genes that are required for siderophore biosynthesis (Carter *et al.*, 2002; Yeoman *et al.*, 2003). It will be interesting to discover how many of the large number of genes whose expression is affected by RirA involve a direct interaction between this regulator and their cognate promoter regions.

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