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Analysis of the *Rhizobium leguminosarum* siderophore-uptake gene *fhuA*: differential expression in free-living bacteria and nitrogen-fixing bacteroids and distribution of an *fhuA* pseudogene in different strains

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A mutation was isolated in the *Rhizobium leguminosarum* gene *fhuA*, which appears to specify the outer-membrane receptor for the siderophore vicibactin. The mutant was defective in iron uptake and accumulated the siderophore vicibactin in the extracellular medium. Expression of *fhuA* was regulated by Fe^{3+}, transcription being higher in iron-depleted cells. Transcription of *fhuA* was independent of a functional copy of *rpoI*, a neighbouring gene that specifies a putative ECF σ factor of RNA polymerase and which is involved in siderophore production in *Rhizobium*. Mutations in *fhuA* did not detectably affect symbiotic N_2 fixation on peas. An *fhuA*::*gus* fusion was expressed by bacteria in the meristematic zone of pea nodules but not in mature bacteroids. Some other strains of *R. leguminosarum* also contain a pseudogene version of *fhuA*. The sequences of some of these and the 'real' *fhuA* genes were determined.

Keywords: ECF σ factor, *fhu* genes, iron-mediated regulation, pseudogene, rhizobia, siderophores

INTRODUCTION

Apart from the lactobacilli, bacteria need iron, which they use as a component of many enzymes. In many biologically relevant conditions, iron exists in the extremely insoluble oxidized ferric form. Therefore, many bacteria make siderophores, small organic molecules that they excrete and which bind Fe^{3+}. The Fe–siderophore complexes are internalized by dedicated transport systems (Braun *et al*., 1998; Crosa, 1997). Since the enzyme complex nitrogenase comprises iron-containing proteins, diazotrophs have a particularly high demand for iron. In the case of the root-nodule bacteria known as the rhizobia, which fix N_2 symbiotically, the requirement must also be satisfied in competition with the host plant; note that the most abundant single plant protein in nodules is the iron-containing leghaemoglobin (see Fett *et al*., 1998).

Rhizobium leguminosarum bv. *viciae*, the symbiont of peas, lentils, vetches and some beans, makes vicibactin, a cyclic trihydroxamate siderophore with three residues each of N^2-acetyl-N^5-hydroxy-D-ornithine and D-hydroxybutyrate (Dilworth *et al*., 1998). Different rhizobia make other hydroxamates (Persmark *et al*., 1993), catechols (Roy *et al*., 1994), citrate (Guerinot *et al*., 1990) or anthranilate (Barsomonian *et al*., 1992). In some cases, rhizobial mutants defective in siderophore synthesis fix N_2 normally (Reigh & O'Connell, 1993; Fabiano *et al*., 1995), but in others, Sid^- mutants fail to fix N_2 symbiotically (Barsomian *et al*., 1992). Yeoman *et al*. (1997) found that *cyc* (*ccm*) mutants of *R. leguminosarum* bv. *viciae* which are defective in cytochrome *c* maturation were, for reasons that are not clear, also compromised for vicibactin synthesis. Such mutants

Abbreviations: CAS, chrome azurol sulphonate; ECF, extracytoplasmic factor; NTA, nitrilotriacetate; X-Gluc, 5-bromo-4-chloro-3-indolyl β-D-glucuronide.

The GenBank/EMBL/DDBJ accession number for the sequence determined in this work is AJ238208.

were also Fix$^-$ on peas, due to the defect in electron transport.

Stevens *et al*. (1999) identified some of the *fhu* genes of *R. leguminosarum*. These are homologues of the corresponding genes in (for example) *Escherichia coli* which are involved in the uptake of hydroxamate siderophores. In *E. coli*, FhuA is an outer-membrane receptor, FhuD a periplasmic transporter, FhuB an integral cytoplasmic membrane protein and FhuC an ATPase (Braun *et al*., 1998). In *R. leguminosarum*, *fhuCDB* are in one operon whose expression is enhanced in cells grown in low concentrations of iron. Mutations in *fhuCDB* caused cells to make larger haloes on plates containing the 'universal' siderophore indicator chrome azurol sulphonate (CAS) (Schwyn & Neilands, 1987) and were defective for vicibactin and iron uptake (Stevens *et al*., 1999). These mutants nodulated and fixed N_2 normally on peas, indicating that vicibactin is not important in iron nutrition in bacteroids. It is not known if these bacteria make another, bacteroid-specific siderophore. It may also be the case that bacteroids acquire iron in the ferrous form; although bacteroids can take up both Fe^{2+} and Fe^{3+} iron, the efficiency is greater with the former (LeVier *et al*., 1996; Moreau *et al*., 1998).

In *E. coli*, *fhuA* is in the *fhuACDB* operon. In *R. leguminosarum* strain 8401pRL1JI, there is a different arrangement in which there is a version of *fhuA* oriented divergently from *fhuCDB*. However, this copy of *fhuA* appears to be a pseudogene; it has many stop codons and is not detectably expressed (Stevens *et al*., 1999). LeVier & Guerinot (1996) identified a gene, *fegA*, in *Bradyrhizobium japonicum* which was a homologue of *fhuA* and which specified an outer-membrane protein, made in response to iron deprivation.

A functional *fhuA* gene of *R. leguminosarum* has now been discovered and is described here. The effects of iron availability and of the regulatory genes *rpoI*, *fur* and *feuQ* on its transcription are described as are its expression in pea root nodules. We also looked for the presence of *fhuA* and its pseudogene version, *ψfhuA*, in a number of field isolates of *R. leguminosarum*.

METHODS

Media and bacterial growth conditions. Strains and plasmids used in this study are shown in Table 1. Strains of *E. coli* and *R. leguminosarum* were grown routinely as described by Beringer (1974). In high-iron medium, $FeCl_3$ (20 μM) was added; in low-iron medium, there was no added iron, but, instead, 2,2′-dipyridyl (20 μM) was present. Peas were inoculated, grown and assayed for N_2 fixation by C_2H_2 reduction as in Beynon *et al*. (1980). Qualitative CAS tests on agar plates were done as described by Yeoman *et al*. (1997).

Enzyme assays. β-Galactosidase and β-glucuronidase assays were done as described by Rossen *et al*. (1985) and by Wilson *et al*. (1992), respectively. Nodules were stained for β-glucuronidase activity *in situ* with 5-bromo-4-chloro-3-indolyl β-D-glucuronide (X-Gluc) (Jefferson *et al*., 1987).

***In vivo* genetic manipulations.** Plasmids were transferred by conjugation into *R. leguminosarum* using the helper plasmid pRK2013 (Figurski & Helinski, 1979). Strain 8401 was mutagenized with Tn*gus* by using it as a recipient in a conjugational cross with *E. coli* strain MM294, which contains a derivative of the plasmid pRK600 into which the transposon Tn*gus* has been inserted (Sharma & Signer, 1990). Since pRK600 is mobilizable into *Rhizobium* but fails to replicate in that host, it acts as a 'suicide' plasmid. Thus, by selecting Kan^r transconjugants (specified by Tn*gus*), derivatives of strain 8401 with random insertions of the transposon into the genome were obtained, at frequencies of approximately 10^{-6}. Kan^r colonies were picked to minimal (Y) medium containing CAS. Transduction of Tn*gus* from mutant A691 was done, using rhizobiophage RL38, as described by Buchanan-Wollaston (1979).

***In vitro* DNA manipulations.** Routine transformations, restriction digestions, ligations, Southern blotting and hybridization were done essentially as described by Downie *et al*. (1983). *R. leguminosarum* genomic DNA was isolated using a Promega genomic preparation kit. Sequencing was done by the dideoxy chain-termination method, in some cases by MWG Ltd, Germany. Data were analysed with the DNA-Star package. Searches of databases used BLAST in the EGCG package. The primers used to amplify the two *fhuA* genes were: *fhuA*, 5′-TCCATAGGTTCCGCCCGCATCCGT-3′ and 5′-TTTCGACGATGTGATAGGCGACCG-3′; *ψ*fhuA, 5′-GGAGCAGATCGGCAAGGTCGGCGTG-3′ and 5′-CGCCGATCGCCGTAATATTCTGTGC-3′. The primers used to amplify the *fhuA* promoter region were: 5′-CGCAGATCTTCGCAGCCATCGAGGGGGC-3′ and 5′-CGCGCATGCCGTAATTGATATAGGGCTGGC-3′.

Iron uptake. Uptake of iron from ^{55}Fe-NTA (prepared with $^{55}FeCl_3$ and sodium nitrilotriacetate) was measured as described by Yeoman *et al*. (1997). Cells were grown in minimal (Y) medium with $FeCl_3$ (20 μM) or in the absence of added iron but with 2,2′-dipyridyl (20 μM). Vicibactin was identified by electrospray mass spectroscopy as described by Yeoman *et al*., 1999.

RESULTS

Isolation of a siderophore-overproducing mutant

Following mutagenesis of *R. leguminosarum* strain 8401 with Tn*gus* (see Methods), one mutant, termed A691, with a larger halo on CAS plates (the diameter was about twice that of the wild-type) was isolated following the screening of approximately 4000 transconjugants. This phenotype was similar to that of *fhuCDB* mutants that were defective in vicibactin uptake (Stevens *et al*., 1999). However, we found that A691 was not corrected by pBIO400, a cosmid from a pLAFR1-based gene library of *R. leguminosarum* DNA containing cloned *fhuCDB* (Stevens *et al*., 1999). The mutation from A691 was transduced into strain 8401, selecting kanamycin resistance. All the transductants co-inherited the CAS phenotype of A691, demonstrating that Tn*gus* caused this phenotype. One transductant, A775, was chosen for further study.

It was shown that A775 was defective in iron uptake. Cells were grown in low-iron medium and exposed to ^{55}Fe-NTA. No detectable uptake of this substrate was observed (<2% of the wild-type value), over a period of

Table 1. Strains and plasmids

	Relevant properties	Source or reference
R. leguminosarum		
8401	Lacks sym plasmid, also Str^r	Lamb *et al.* (1982)
8401pRL1JI	*R. leguminosarum* bv. *viciae*; contains sym plasmid pRL1JI	Downie *et al.* (1983)
J100	*feuQ* mutant of 8401pRL1JI	Yeoman *et al.* (1997)
A691	8401 *fhuA1*::Tn*gus* (obtained by Tn*gus* mutagenesis)	This work
A775	8401 *fhuA1*::Tn*gus* (transduced from A691)	This work
J253	A775(pRL1JI)	This work
J256	*rpoI* mutant of 8401pRL1	This work
BA7, BA11, BB18, BC1, FIII-70, FIII-72, RES-2, RES-6, RES-7 and SC1	Field isolates of *R. leguminosarum* bv. *viciae*	Rigottier-Gois *et al.* (1998)
E. coli		
803	Used for transformation of large plasmids	Wood (1966)
MM294	Strain harbouring pRK600	Sharma & Signer (1990)
Bacteriophage		
RL38	Used for generalized transduction	Buchanan-Wollaston (1979)
Plasmids		
pUC18	Sequencing vector	Messing *et al.* (1983)
pBluescript	Cloning vector	Stratagene
pRK2013	Mobilizing plasmid for conjugation; Kan^r	Figurski & Helinski (1979)
pMP220	Wide-host-range promoter-probe vector; Tet^r	Spaink *et al.* (1987)
pRK415	Wide-host-range cloning vector; Tet^r	Keen *et al.* (1988)
pRK600	Suicide plasmid for delivery of Tn*gus*	Sharma & Signer (1990)
pBIO1096	pLAFR1-based cosmid containing approx. 25 kb *R. leguminosarum* DNA, including *fhuA* and *rpoI*; Tet^r	Yeoman *et al.* (1999)
pBIO1097	*Pst*I fragment containing *fhuA* cloned in pRK415	This work
pBIO1111	*fhuA* PCR promoter fragment, cloned in pMP220	This work
pBIO929	Approx. 30 kb of *R. leguminosarum* DNA cloned in pLAFR1; contains *fur* gene	deLuca *et al.* (1998)
pIJ9116	Part of Tn*gus* plus flanking *fhuA* DNA, cloned in pBluescript	This work

15 min. It was also confirmed that the extra CAS-staining material was vicibactin, not some novel siderophore. Cells of A775 were grown and the cell-free growth medium assayed for hydroxamate. Strain A775 contained approximately four times more hydroxamate than did the wild-type and it was confirmed by electrospray mass spectroscopy that this hydroxamate corresponded to vicibactin.

Cloning and analysis of the *fhuA* gene

To locate the Tn*gus* insertion precisely, DNA was isolated from strain A775 and digested with *Eco*RI. Fragments were ligated to pBluescript and used to transform *E. coli*, selecting Kan^r transformants. By such means, part of Tn*gus*, containing Kan^r and *gus*, together with *Rhizobium* genomic DNA immediately upstream of the *gus* reporter, was cloned to form pIJ9116. The DNA adjacent to *gus* was sequenced; this indicated that the transposon was located in DNA whose deduced protein product had similarity to the C-terminus of FhuA of *E. coli* and other bacterial hydroxamate receptors (Table 2; and see below). Significantly, this 3′ end of an *fhuA* homologue was similar in sequence to the corresponding region of the pseudogene version, *ψfhuA*, identified by Stevens *et al.* (1999) in the same strain of *R. leguminosarum*. However the sequences were not identical (see below). Sequencing also showed

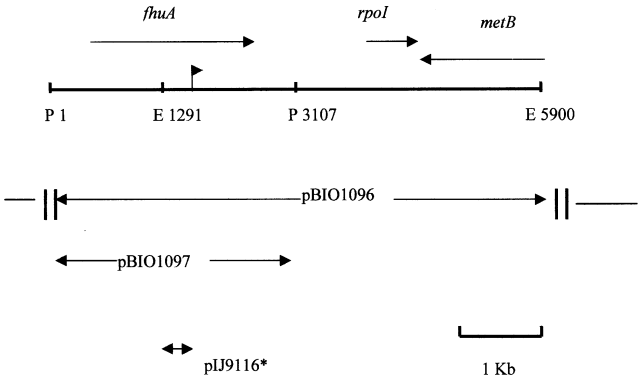

Fig. 1. Diagram of the *fhuA* region of *R. leguminosarum*. The approximate locations and directions of transcription of the genes in the *rpoI* region are indicated. Restriction sites for *Eco*RI (E) and *Pst*I (P) are shown, as are the dimensions of the cloned DNA in plasmids pBIO1096 and pBIO1097. The 'flag' above the restriction map indicates the location of the Tn*gus* insertion in strain A775. The asterisk indicates that the cloned DNA in pIJ9116 also contains a piece of Tn*gus*.

that in A775, the *gus* reporter Tn*gus* was in the same orientation as *fhuA*.

Yeoman *et al*. (1999) identified a putative ECF regulatory gene, *rpoI*, in *R. leguminosarum* bv. *viciae*. During the course of other studies on *rpoI*, the region upstream of the *rpoI* coding sequence had been determined (K. H. Yeoman, unpublished). We noted that this upstream sequence overlapped the 3′ region of *fhuA*, which was sequenced (see above). The *rpoI* gene had previously been cloned as part of a cosmid, termed pBIO1096 (Yeoman *et al*., 1999). We used this cosmid as a source of DNA to confirm the overlap between the part of *fhuA* identified here and the previously identified DNA upstream of *rpoI* (see Fig. 1). pBIO1096 DNA was digested with different restriction enzymes, and following electrophoresis and blotting, was probed with pIJ9116 DNA. A single 3·7 kb *Pst*I fragment hybridized. This fragment was subcloned from pBIO1096 into the wide-host-range vector pRK415 to form pBIO1097, which was then mobilized into strain A775. The transconjugants were restored to wild-type phenotype on CAS plates, showing that this fragment contained a functional *fhuA* gene. This fragment was sequenced; it was found to contain the whole of *fhuA*, plus 333 bp of upstream sequence (accession no. AJ238208).

The deduced FhuA protein had a molecular mass of 79·5 kDa, and was similar throughout its length to FhuA proteins of other bacteria (Table 2) the greatest similarity, overall, being to FegA of *B. japonicum* (34·8 % identity). The other proteins in the FhuA family are all predicted to be in the outer membrane and most have a typical signal sequence. The predicted *fhuA* gene product from *R. leguminosarum* has a potential signal sequence but it is not entirely typical (MARVFLNVSN-NVSRIYRDSLFVTTAIVLIGIAASPAASQS). Thus, the first 18 residues are fairly hydrophilic and include three positively charged residues (R) and one acidic residue (D), followed by a hydrophobic stretch and a potential signal peptidase cleavage site (PAA/S). This predicted 40-residue signal sequence is somewhat larger than normal and the presence of the aspartate is atypical. However, the net positive charge of the N-terminal domain followed by the hydrophobic residues may be consistent with this region acting as a signal peptide. TonB-dependent outer-membrane proteins, such as FhuA, share amino acid sequences, termed the TonB boxes I, II and III (see Postle, 1999). FhuA of *R. leguminosarum* has a potential Ton box I (EVPRS) near the N-terminus and putative Boxes II and III are present between amino acid residues 693–699 and 167–190, respectively.

Table 2. Comparison of *R. leguminosarum* FhuA and ψFhuA with other proteins of this family

Protein	Accession no.	Percentage identity	
		FhuA*	ψFhuA†
Rhizobium leguminosarum FhuA	AJ238208		32·7
Bradyrhizobium japonicum FegA	U61401	34·8	35·0
Erwinia amylovora FoxR	g2760450	31·9	31·3
Erwinia chrysanthemi FctA	X87967	31·7	35·2
Yersinia enterocolitica FoxA	X60447	30·3	32·9
Pseudomonas aeruginosa FptA	P42512	30·0	29·7
Escherichia coli FhuA	M12486	29·9	41·1
Escherichia coli FepA	M13748	20·7	28·0

*Figures show percentages of identical amino acids over the entire length of the *R. leguminosarum* FhuA protein.

†Figures show percentages of identical amino acids in the C-terminal part of *R. leguminosarum* ψFhuA shown in Fig. 3.

In the region 5′ of *fhuA* there were no obvious regulatory sequences (e.g. a *fur* box) and the promoter of this gene has not yet been identified. A gap of 1·5 kb separates the 3′ end of *fhuA* and the start of *rpoI*. Within this DNA there was no ORF larger than 300 bp and there was no homology of this DNA nor any potential peptide products to sequences in databases. However, this is a rather large intergenic space, and it may be that it does contain a short gene of unknown function. In the intergenic region, 57 bp 3′ of *fhuA* is a perfect inverted repeat, (5′-**CCGTCGCCCACCAGGCC**CGTCGACC-TCGAC**GGCCTGGTGGGCGACGG**-3′), which might act as a ρ-independent transcriptional terminator.

Expression of *fhuA*: effects of iron, *rpoI*, *feuQ* and *fur*

The *fhuCDB* operon of *R. leguminosarum* is expressed at higher levels in cells that are depleted for iron than in those that are replete for the metal (Stevens *et al*., 1999). A derivative of the *fhuA*::*gus* mutant strain A775 containing pBIO1097 (to correct the defect in iron uptake of strain A775 itself), was grown in high- and low-iron media (see Methods) and assayed for β-glucuronidase. As with *fhuCDB*, expression was higher in the latter than the former medium, the activity being 54 ± 12 and 858 ± 67 Miller units, respectively.

We had previously identified two *R. leguminosarum* genes, *feuQ* and *rpoI*, which are believed to be regulatory and affect iron uptake. Yeoman *et al*. (1997) showed that a mutation in *feuQ* (which is likely to be a sensor in another representative of the two-component family of transcriptional regulators) severely affected iron uptake, although siderophore production appeared to be unaltered. Mutations in *rpoI*, which is downstream of *fhuA*, nearly abolish vicibactin production and the presence of cloned *rpoI* enhances siderophore production in *R. leguminosarum*.

To measure the effects of *feuQ* and *rpoI* on *fhuA* expression, an *fhuA*::*lacZ* transcriptional fusion plasmid was made as follows. A 1 kb PCR fragment, containing 470 bp of the N-terminal coding region of *fhuA* plus 530 bp upstream of *fhuA* was made, using the primers shown in Methods, and with pBIO1096 as template. This fragment was cloned first into pUC18 and thence into the *Eco*RI–*Sph*I sites of the wide host-range promoter-probe plasmid pMP220 to form pBIO1111. This plasmid was then mobilized into wild-type strain 8401pRL1JI and the *feuQ* and *rpoI* mutant derivatives, J100 and J256, respectively. The transconjugants were grown in high- and low-iron media and were assayed for β-galactosidase. As with the *fhuA*::*gus* fusion, it was found that addition of Fe^{3+} to the growth medium reduced expression of the fusion. This was true for the wild-type background (211 Miller units in high-iron and 1555 units in low-iron medium) and in the two mutants J100 (216 and 1455 units) and J256 (188 and 1318 units). These results showed that neither *rpoI* (strain J256) nor *feuQ* (strain J100) is required for transcription of *fhuA*, nor do they mediate the iron-dependent control of its expression. It had also been shown previously that neither *rpoI* nor *feuQ* had any detectable effect on expression of an *fhuB*::*lacZ* fusion in either high- or low-iron media (Stevens *et al*., 1999; Yeoman *et al*., 1999).

deLuca *et al*. (1998) described a homologue of the 'global' regulator *fur* in *R. leguminosarum* but could not obtain a knockout mutation in it, suggesting that this gene is essential. Nevertheless, since *fur* regulates expression of the *fhu* genes of other bacteria in an iron-dependent way (Crosa, 1997), we examined the effects of *fur* on expression of the *fhuA*::*gus* fusion in strain A775 by conjugating into it plasmid pBIO929, which contains *fur* of *R. leguminosarum*. This derivative was grown in high- and low-iron media and assayed for β-glucuronidase; it had no effect on the *fhuA*::*gus* expression in either medium. However, in the absence of a knockout mutant, we cannot be sure whether *fur* has a role in regulating *fhuA* transcription or not.

fhuA and symbiotic nitrogen fixation

The original *fhuA* mutant, A775, was derived from *R. leguminosarum* strain 8401, which lacks a symbiotic plasmid and so fails to nodulate. To examine the effects of the *fhuA*::*gus* mutation on nodulation, peas were inoculated with strain J253, a derivative of A775 into which plasmid pRL1JI had been introduced by conjugation. Judged by the numbers and sizes of the nodules, their time of appearance and the levels of C_2H_2 reduction, J253 appeared to be unaffected in symbiotic N_2 fixation. Bacteria isolated from these nodules were found to retain the large-halo phenotype of the input *fhuA* inoculant and were all Kan^r. This Nod^+ Fix^+ phenotype is similar to what is seen with *fhuCDB* mutants (Stevens *et al*., 1999) and points to the fact that the *fhu* system of Fe^{3+} uptake is unimportant in N_2-fixing bacteroids. Consistent with this were the observations obtained with nodules stained with X-Gluc. The only part of the nodule with significant β-glucuronidase activity was near the meristem, where non-differentiated bacteria, some still in infection threads, are located. In the zone containing mature, N_2-fixing bacteroids, no staining was seen (Fig. 2).

Since these nodules were induced by mutants that were defective in vicibactin uptake, their pattern of expression of *fhuA*::*gus* might not reflect the normal situation. Therefore, pBIO1097, which contains cloned *fhuA*, was mobilized into J253 and transconjugants were used to inoculate peas. The same pattern of staining with X-gluc of the nodules was found as with strain J253 itself (not shown).

Pseudogene ψ*fhuA* occurs in different *R. leguminosarum* strains

Since a mutation in the version of *fhuA* described here causes a defect in iron uptake and a large-halo phenotype on CAS, it is clear that this gene is functional. In

Fig. 2. Pea root nodule containing *fhuA*::*gus* fusion strain A253 and stained with X-gluc. Note that staining for *β*-glucuronidase occurs only at the nodule tip, containing the undifferentiated bacteria. The mature, N_2-fixing zone, nearer the root, has no detectable staining.

```
     *      * *   *** **  ***          ** *
VNPLTLAYDS LGEVTGKGIE LEARAAIADG LDIIAAYTYN   FhuA
TRRSCLRLEQ IGKVGVRGIE LEGKAAINDR VNLTLAYSYW   ψ FhuA
          P1

  *      *  * ** *    * * **     **
HSEV---TGG DNEGNTPAFT PAHVASLWAN YTFQETNPFN   FhuA
DAEIREDGTG GNIGNWPSRV PRHLASARLD YTVPGDGKRG   ψ FhuA

 *  * ****       * ** *        * **   *
GLSVGAGVRY VSENWTDTAN TSKNPSSFYV DASAAYDFGA   FhuA
DLTLGGGVRY IGQTHGDEAN TVSVAAYTLV DAAVSYKVTP   ψ FhuA

        *   *  *          * *    * *  *
IDKNYEGLTA AFNIRNIADQ RDTVCNEGFC YLGQGRNMTA   FhuA
DVTLAVDATN LFD-RKYVA- SSYFCTE--- YYGDRRKVVG   ψ FhuA
      N                      P2

**** *
TLKYRW                                        FhuA
TLKYSW                                        ψ FhuA
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Fig. 3. Comparison of the C-terminal regions of the FhuA and corresponding FhuA gene products of *R. leguminosarum* strain 8401pRL1JI. The deduced amino acid sequences are shown in single-letter code. Asterisks indicate identical residues. The bold '**N**' indicates the difference in the sequences of the ΨFhuA gene product in strains BC1 and RES-6 compared to that sequence in strain 8401pRL1JI. The locations corresponding to those used for generating the primers P1 and P2, used to amplify *ψfhuA*, are shown.

contrast, the *ψfhuA* pseudogene that is adjacent to *fhuCDB*, but which is unlinked to the functional *fhuA* gene identified here, is a non-functional pseudogene and is not expressed (Stevens *et al*., 1999).

A comparison of parts of the C-terminal regions of FhuA and *ψ*FhuA is shown in Fig. 3. The similarity is no greater than that between *R. leguminosarum* and members of the FhuA family of proteins in other bacteria (Table 2). We wished to see if genes corresponding to *ψfhuA* were widespread in strains of *R. leguminosarum*. Probes corresponding to parts of *fhuA* and *ψfhuA* were made by PCR using as templates pBIO1096 and pBIO400, which respectively contain the two versions of the gene. The locations of the primers used for *ψfhuA* are shown in Fig. 3; those used for *fhuA* are given in Methods and are located in the 5′ half of that gene.

Two PCR products of the expected sizes were generated from *fhuA* and *ψfhuA*. Each product was then used to probe DNA obtained from 10 field isolates of this species. These strains had been shown from RFLP to be distinct from each other and from 8401pRL1JI (Rigottier-Gois *at al*., 1998). Genomic DNAs were digested with *Eco*RV and *Pst*I and, after electrophoresis, were probed separately with the two PCR ^{32}P-labelled products. With the *fhuA* probe, bands hybridizing as intensely as with strain 8401pRL1JI were seen for all 10 strains (Table 3). These exhibited some RFLP, different patterns being obtained which were consistent with the relatedness of the strains (Rigottier-Gois *et al*., 1998) (Table 3). With the *ψfhuA* probe, strains BC1 and RES-6 hybridized strongly and to the same-sized bands as with 8401pRL1JI. Strain BB18 gave a very weak signal, the band being larger than for 8401pRL1JI; seven strains had no detectable signal with the *ψfhuA* probe. The two strains hybridizing to *ψfhuA* had similar *fhuA* RFLP patterns to each other and to strain 8401pRL1JI (Table 3). Following hybridization of the *Eco*RV-digested DNAs with the *ψfhuA* probe, the filter was stripped and reprobed with *fhuA* DNA. The original bands did not reappear, but 'new' bands, corresponding to those expected for *fhuA*, were seen (Table 3). Thus, there was no cross-hybridization between *ψfhuA* and *fhuA* in the strains used here.

The *ψfhuA* primers were used to amplify the corresponding DNA from the genomes of strains BC1 and RES-6 and the resulting fragments were sequenced. These products were identical to each other and differed by one nucleotide (causing a D to N substitution in the protein) from that of strain 8401pRL1JI (Fig. 3); by chance this difference resulted in the loss of a *Sal*I site in strains RES-6 and BC1. It was confirmed that there was a *Sal*I RFLP at this location. We failed to get a genomic PCR product from strain BB18, which showed weak hybridization to *ψfhuA*.

Table 3. Sizes (kb) of *Eco*RV and *Pst*I genomic fragments hybridizing to *fhuA* and *ψfhuA* probes in field isolates of *R. leguminosarum* bv. *viciae*

	Strain										
	8401pRL1	**BA7**	**BA11**	**BB18**	**BC1**	**FIII-70**	**FIII-72**	**RES-2**	**RES-6**	**RES-7**	**SC1**
Genomic DNA digested with *Eco*RV											
Probe *ψfhuA*	2·0	–	–	2·5*	2·0	–	–	–	2·0	–	–
Probe *fhuA*	7·0	1·5	1·5	1·5	7·0	6·0	1·5	1·5	7·0	6·0	1·5
	3·5	0·7	0·7	0·7	3·5	3·1	0·7	0·7	3·5	3·1	0·7
	0·7				0·7	0·7			0·7	0·7	
Genomic DNA digested with *Pst*I											
Probe *fhuA*	2·6	3·1	3·1	3·1	7·0	4·0	3·1	3·1	2·6	4·0	3·1
		1·7	1·3	1·7	3·0	1·3	1·7	1·7		1·3	1·3
			0·7		2·3						0·7

–, No detectable hybridization.

* Very faint hybridization.

DISCUSSION

We have identified a functional copy of *fhuA* which is homologous to *fhuA* genes which, in other bacteria, encode the receptor for hydroxamate siderophores (see Braun *et al*., 1998). In *B. japonicum*, an FhuA homologue, termed FegA, was shown to be present in enhanced levels in bacteria depleted of iron (LeVier & Guerinot, 1996).

Using transcriptional fusions to *gus* and to *lacZ*, we found that *fhuA* of *R. leguminosarum* was transcribed at elevated levels in cells starved of iron. Stevens *et al*. (1999) had similarly found that the unlinked *fhuCDB* operon of *R. leguminosarum* was Fe^{3+} regulated. We do not know, however, what regulatory gene mediates this iron-dependent expression. It is apparent from the results obtained here that two regulatory genes, *rpoI* and *feuQ*, which affect iron uptake in *R. leguminosarum*, are not involved. Given the adjacent locations of *rpoI* and *fhuA*, together with the facts that mutations in *rpoI* abolish siderophore synthesis and that overexpression of *rpoI* causes enhanced production of vicibactin (Yeoman *et al*., 1999), we were surprised at the lack of interaction between *fhuA* and *rpoI*. At present, the 'target' gene(s) that require the RpoI σ factor for their transcription remains to be identified.

An *fhuA*::*gus* fusion was used to show that although *fhuA* is expressed in undifferentiated bacteria in the nodule, the mature N_2-fixing bacteroids do not transcribe this gene at detectable levels. The basis of this switch-off regulation is not known. Several genes that are expressed in free-living rhizobia are quiescent in bacteroids; these include the *pss* (*exo*) genes for polysaccharide synthesis (Latchford *et al*., 1991), the *amtB* gene that is involved in ammonium transport (Tate *et al*., 1999), and some *nod* genes required for the early steps in the infection process (Schlaman *et al*., 1991; Marie *et al*., 1992). The mechanisms involved in the down-regulation of genes in bacteroids have received little attention and it remains to be seen if there is some 'global' control or if individual genes have specific shutdown systems. The finding that *fhuA* is not expressed in bacteroids is consistent with the lack of symbiotic defects found with various *fhu* mutants (this study; Stevens *et al*., 1999). To date, the only *R. leguminosarum* mutants as yet identified that are defective both in siderophore synthesis and in N_2 fixation are the *cyc* (*ccm*) mutants described by Yeoman *et al*. (1997). In these cases, it seems almost certain that it is the respiratory defect rather than that in siderophore synthesis that is responsible for the symbiotic phenotype (Delgado *et al*., 1995). The negative results with defined *fhu* mutants strongly indicate that vicibactin is not used for iron uptake in *R. leguminosarum* bacteroids. It may be that there is another, unknown bacteroid-specific siderophore system. Alternatively, there is circumstantial evidence that bacteroids acquire iron in the reduced, ferrous form. Bacteroids of *B. japonicum* in soybean nodules can import both Fe^{3+} and Fe^{2+}, but the uptake of the latter is more efficient (Moreau *et al*., 1998). However, in the absence of (*feo*) mutants that are defective in Fe^{2+} uptake, it is impossible to know the relative importance of the two forms of iron in bacteroid nutrition.

Stevens *et al*. (1999) identified a pseudogene version of *fhuA*, next to the functional *fhuCDB* genes. In that study, no hybridization to any other DNA was observed when a probe spanning *ψfhuA* was used. It is clear, though, that *R. leguminosarum* strain 8401pRL1JI does contain a functional gene that is unlinked to *ψfhuA*; however, a comparison of the sequences of *fhuA* and *ψfhuA* in this strain shows that there is DNA and protein homology in only limited areas. The similarity of the potential products of the *fhuA* and *ψ*fhuA sequences in *R. leguminosarum* strain 8401pRL1JI is no greater than that found between the FhuA of this strain and those of other bacteria. This suggests that these two genes did not arise via recent gene duplication followed

by limited divergence. Rather, it points to *fhuA* and *ψfhuA* having had quite different origins, one, perhaps, having been acquired by gene transfer.

It is apparent that other different field isolates of *R. leguminosarum* contain homologues of both the functional and the pseudogene versions of *fhuA*. However, the two strains that contained a close homologue of *ψfhuA* appeared to be closely related to each other and to strain 8401pRL1JI as judged from their RFLP patterns at other loci (Rigottier-Gois *et al*., 1998; this study). In these two strains, the sequenced regions of the *ψfhuA* homologues were identical to each other and differed in only one base pair from the allele in 8401pRL1JI. We were surprised that a pseudogene version of the gene differed so little in different strains since, by definition, such genes are not subject to the constraints that are required to maintain gene function. It will be of interest to know the precise sequences of the regions around the pseudogene versions of *fhuA* in different strains of *R. leguminosarum*. Are they in the same relative positions in the chromosomes? How large are the regions that distinguish these pseudogene regions from those in the strains that do not harbour them (or which, perhaps, contain different versions of *fhuA* pseudogenes)?

Pseudogenes are relatively rare in prokaryotes. It was noted by Stevens *et al*. (1999) that in bacteria, the deduced original products of several pseudogenes, including *R. leguminosarum ψfhuA*, are located at the cell surface. FhuA of *E. coli* is a receptor for several coliphages, colicins and at least one antibiotic (Killman & Braun, 1992; Killman *et al*., 1995). It may be that there is particularly strong selection pressure to lose versions of such cell-surface proteins that can act as targets for such antimicrobial agents. The finding here that pseudogenes exist in only a minority of strains of *R. leguminosarum* suggests that such selection pressure may be intermittent and not uniform in different populations.

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