The *fhu* genes of *Rhizobium leguminosarum*, specifying siderophore uptake proteins: *fhuDCB* are adjacent to a pseudogene version of *fhuA*

James B. Stevens, Robert A. Carter, Haitham Hussain, Kerry C. Carson, Michael J. Dilworth and Andrew W. B. Johnston

A mutant of *Rhizobium leguminosarum* was isolated which fails to take up the siderophore vicibactin. The mutation is in a homologue of *fhuB*, which in *Escherichia coli* specifies an inner-membrane protein of the ferric hydroxamate uptake system. In *Rhizobium*, *fhuB* is in an operon *fhuDCB*, which specifies the cytoplasmic membrane and periplasmic proteins involved in siderophore uptake. *fhuDCB* mutants make vicibactin when grown in Fe concentrations that inhibit its production in the wild-type. Nodules on peas induced by *fhuDCB* mutants were apparently normal in N₂ fixation. Transcription of an *fhuDCB-lacZ* fusion was Fe-regulated, being approximately 10-fold higher in Fe-depleted cells. Downstream of *fhuB*, in the opposite orientation, is a version of *fhuA* whose homologues in other bacteria specify hydroxamate outer-membrane receptors. This *fhuA* gene appears to be a pseudogene with stop codons and undetectable expression.

Keywords: *fhu* genes, iron-mediated regulation, pseudogene, *Rhizobium leguminosarum*, siderophores

**INTRODUCTION**

With the possible exception of lactobacilli, iron is essential for all organisms, being a component of many proteins. Although relatively abundant, in its normal Fe³⁺ form, it is very insoluble at normal pH and pO₂. Therefore, many bacteria make and export low-Mᵣ compounds, known collectively as siderophores, which bind avidly to Fe³⁺. These Fe-siderophore complexes are bound to specific receptors at the bacterial cell surface, before being internalized by dedicated transport systems (see Guerinot, 1994; Braun et al., 1998; Crosa, 1997). Iron is of particular importance to N₂-fixing bacteria since it is a component of nitrogenase and electron donors specifically involved in N₂ reduction. Also, in the case of the symbiosis between legumes and bacteria known collectively as the rhizobia, the bacteria in N₂-fixing root nodules must acquire their Fe from the host, in which Fe-containing leghaemoglobin is very abundant (Guerinot, 1991).

Different rhizobial species and strains make various siderophores, including rhizobactin, a carboxylate (Smith et al., 1985), hydroxamates (Dilworth et al., 1998; Persmark et al., 1993), catechols (Patel et al., 1988; Modi et al., 1985; Roy et al., 1994), citrate (Guerinot et al., 1990) and anthranilate (Rioux et al., 1986; Barsomian et al., 1992). Mutants that do not make or export siderophores have been isolated but their symbiotic phenotypes have, apparently, not been consistent. Thus, some mutants of *Sinorhizobium* (formerly *Rhizobium*) *meliloti* which fail to make rhizobactin (Barton et al., 1996b) or anthranilate (Barsomian et al., 1992) are defective in N₂ fixation on alfalfa, but others are unaffected (Fabiano et al., 1995; Reigh & O'Connell, 1993).

A *Rhizobium leguminosarum* mutant defective in siderophore synthesis (Yeoman et al., 1997) failed to fix N₂ in pea nodules, and was also defective in synthesizing all c-type cytochromes. The mutation was in the *cycHJKL* operon, which in R. *leguminosarum*, S.
meliloti and Bradyrhizobium japonicum is required for maturation of c-type cytochromes; it is, in fact, the respiratory defect that causes the Fix- phenotype (Delgado et al., 1995; Kereszt et al., 1995; Ritz et al., 1995). Other, uncharacterized siderophore-defective, Fix- mutants may therefore have lesions in cyc; i.e. their symbiotic defects are not due to loss of siderophore production per se.

The trihydroxamate siderophore, vicibactin, made by R. leguminosarum is a cyclic molecule, with three residues each of N²-acetyl-N²-hydroxy-d-ornithine and D-hydroxybutyrate, in alternate ester and peptide bonds (Dilworth et al., 1998). Here, we describe a mutant of R. leguminosarum which overproduces vicibactin. It fails to take up label when presented with 55Fe-vicibactin and is mutated in an operon whose products are similar to the Fhu proteins of Escherichia coli, which are involved in the uptake of ferric hydroxamates. LeVier & Guerinot (1996) identified an outer-membrane protein, FegA, of B. japonicum which is similar to the FhuA receptor of E. coli and other bacteria.

METHODS

Bacterial strains, plasmids and growth conditions. For strains and plasmids, see Table 1. Growth conditions for Rhizobium and E. coli were as in Beringer (1974). Peas were inoculated, grown and assayed for N₂ fixation as described by Beynon et al. (1980). β-Galactosidase activities were assayed as described by Rossen et al. (1985).

Growth in Fe-deficient media. To lower the concentration of available Fe, 4,4'-dipyridyl (20 μM) was added to minimal Y medium which lacked exogenously added Fe. Cultures for measurement of rates of siderophore production and 55Fe uptake were grown in the minimal salts medium of Brown & Dilworth (1975) as described by Carson et al. (1994) with 20 mM mannitol as C source, but with NH₄Cl at 10 mM. The concentration of added Fe (μM) is indicated by a number after MSN; thus MSN-20 indicates 20 μM added Fe. Bacteria were grown in flasks containing 20% of their volume of medium on a rotary shaker (200 r.p.m.) at 28 °C. Turbidity was measured at 600 nm; if necessary, cells were diluted with MSN-0 so that the OD₆₀₀ was in the range 0-1-0-5. In experiments to measure siderophore production rates, OD₆₀₀ and vicibactin concentrations were measured as a time-course extending into stationary phase. Vicibactin concentrations were then plotted against the accumulated value of ΔOD₆₀₀ dt, approximated as the area under the OD curve (de Hollaender & Stouthamer, 1979), the rate of synthesis being derived from the slope of the line. Rates are given on a dry weight basis, using a value of 0-329 mg dry weight mL⁻¹ for a culture with an OD₆₀₀ of 1.

Siderophore analysis. Siderophore production was analysed using Chrome azurol S (CAS) agar plates (Schwyn & Neilands, 1987). Fe-starved cells (10 μl at OD₆₀₀ 0-7) were spotted onto Y minimal agar medium containing CAS and incubated at 28 °C for 3 d. Siderophore production was seen as an orange halo around the bacteria. Vicibactin concentrations were calculated from their absorbance at 450 nm in 3-3 mM Fe(ClO₄)₃ in 0-073 M HClO₄ (Carson et al., 1992) using an experimentally determined molar absorption coefficient of 1510 M⁻¹ cm⁻¹ (Carson et al., 1994).

Iron assay. Rhizobium cells were grown in 400 ml volumes of MSM-0 and MSM-20 with the Fe added as ferric EDTA to prevent possible contamination of cells by precipitated Fe. Cells were harvested by centrifugation after reaching stationary phase (2 d for MSM-20 and 4 d for MSM-0), washed twice with a culture volume of sterile water, resuspended in 10 ml deionized water and freeze-dried. Samples of cells (40 mg dry

**Table 1.** Strains and plasmids used in this study

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Characteristics</th>
<th>Reference</th>
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<tr>
<td>Strains</td>
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<tr>
<td>8401pRL1J1</td>
<td>R. leguminosarum bv. viciae; str</td>
<td>Downie et al. (1983)</td>
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<td>8401</td>
<td>8401pRL1JI cured of sym plasmid pRL1JI</td>
<td>Lamb et al. (1982)</td>
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<td>Yeoman et al. (1997)</td>
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<td>8401pRL1JI, fhuB1::TnphoA</td>
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</tr>
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<td>J302</td>
<td>8401 fhuB1::TnSlac</td>
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</tr>
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<td>803</td>
<td>E. coli, used for transformation</td>
<td>Wood (1966)</td>
</tr>
<tr>
<td>A118</td>
<td>E. coli with TnSlacZ</td>
<td>Simon et al. (1989)</td>
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<td>Plasmids</td>
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<td>Vector for R. leguminosarum gene bank</td>
<td>Friedman et al. (1982)</td>
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<td>pRK415</td>
<td>Wide-host-range cloning vector; Tet'</td>
<td>Keen et al. (1988)</td>
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<tr>
<td>pRK2013</td>
<td>Helper plasmid</td>
<td>Figurski &amp; Helinski (1979)</td>
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<tr>
<td>pPH111</td>
<td>Incompatible plasmid used for marker exchange</td>
<td>Beringer et al. (1978)</td>
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<tr>
<td>pRT733</td>
<td>Used for delivering TnphoA</td>
<td>Taylor et al. (1989)</td>
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<td>pUC18</td>
<td>Used for sequencing</td>
<td>Messing et al. (1983)</td>
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<td>pMP220</td>
<td>Promoter-probe vector</td>
<td>Spank et al. (1985)</td>
</tr>
<tr>
<td>pBIO400</td>
<td>pLAFR1-based cosmid, contains cloned fhu genes</td>
<td>This work</td>
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<tr>
<td>pBIO402</td>
<td>16 kb EcoRI fragment from pBIO400 cloned in pRK415</td>
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</tr>
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<td>pBIO403</td>
<td>pBIO400 fhuB1::TnSlac</td>
<td>This work</td>
</tr>
<tr>
<td>pBIO405</td>
<td>pBIO400 fhuC1::TnSlac</td>
<td>This work</td>
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</table>
wt) were digested in 70% (w/w) nitric acid for 4 h at 125 °C, diluted to 10 ml and the solutions assayed for Fe on a Varian Liberty 200 ICP spectrometer.

59Fe uptake. Exponentially growing cells in either MSM-0 or MSM-20 were harvested, washed and resuspended in MSM-0 without a C or N source at approximately 0·11 mg dry wt ml⁻¹. Uptake of 59Fe-vicibactin complex was measured as described by Carson et al. (1994). The stock solutions of 59Fe-vicibactin contained 1 mM 59FeCl₃ (0·9 MBq ml⁻¹) and 5 mM vicibactin in 10 mM HEPES (pH 6·8); stocks of 59Fe-ferric citrate contained 100 mM sodium citrate and 1 mM 59FeCl₃ (0·6 MBq ml⁻¹). The 100-fold excess of citrate ensured formation of ferric citrate complexes (Messenger & Ratledge, 1982). Uptake was initiated by adding 25 ml 59Fe-siderophore complex to 9·975 ml washed cells (final Fe concentration 2·5 μM). Samples (1 ml) were taken at 0, 2, 4, 6, 8 and 10 min; the cells filtered from them were washed, twice with 20 ml neutralized 10 mM nitritotriacetic acid and twice with 20 ml of MSM-0 lacking a C or N source. Assays were done in duplicate and all experiments were repeated at least once. Values were corrected for the binding of the Fe complexes to the filter and the data presented are the means of four values.

In vivo genetic manipulations. Plasmids were transferred to *R. leguminosarum* by conjugation (Buchanan-Wollaston et al., 1980) using the plasmid pRK2013 as a helper in triparental matings (Figurski & Helinski, 1979). Mutagenesis of *R. leguminosarum* 8401pRL1JI was done by using it as a recipient for plasmid pRT733 in a conjugal transconjugant. This plasmid (Taylor et al., 1989) contains a copy of transposon TnphoA. Since this phoA lacks the 5' leader that allows the alkaline phosphatase (AP) to be targeted to the periplasm (in which AP has activity) only in-frame insertions into genes whose products are exposed to the periplasm will generate fusions with AP activity (Manoil & Beckwith, 1985). pRT733 fails to replicate in *Rhizobium*, so selection for Kan' (specified by TnphoA) results in mutants where TnphoA has inserted randomly in the genome (Long et al., 1988).

For TnSlacZ mutagenesis of pBIO400, the plasmid was transformed into *E. coli* AI18, which has TnSlacZ in the chromosome (Simon et al., 1989). This strain was used in a triparental mating (with pRK2013) with *R. leguminosarum* bv. *viciae* 8401pRL1JI as recipient and selection on TY medium containing streptomycin (8401pRL1JI), kanamycin (TnSlacZ) and tetracycline (pBIO400). Transconjugants arose at frequencies of approximately 10⁻⁴ per recipient, these being due to the transfer of derivatives of pBIO400 into which TnSlacZ had transposed. Mutagenized plasmids were isolated from R. leguminosarum en masse, introduced into *E. coli* 803 by transformation, then retransformed to mutant strain J300 to identify any that no longer complemented its siderophore defect. For these, the sites of insertion and the orientation of the TnSlacZ were determined. The *flu*: TnSlacZ mutations in pBIO400 were introduced into the genome by marker exchange (Ruvkun & Ausubel, 1981) using the P1 incompatibility plasmid pPH1JI to eliminate the pBIO400 (Downie et al., 1983).

DNA manipulations. Restriction enzymes and DNA ligase were used according to the manufacturer's instructions. Transformation of *E. coli* was done according to Maniatis et al. (1982). DNA sequencing was done using an ALF (Pharmacia) semi-automated sequencer according to the manufacturer's instructions. The data were analysed using programs in the DNA-Star package. Searches of databases used BLAST in the EGGCG package.

RESULTS AND DISCUSSION

Isolation of siderophore-overproducing *Rhizobium* mutant

In a search for mutants altered in siderophore secretion or uptake, *R. leguminosarum* bv. *viciae* 8401pRL1JI was mutagenized with TnphoA (see Methods) and derivatives plated on media containing XP, the chromogenic indicator for AP, the product of phoA. Of 122 blue (AP-positive) TnphoA-containing derivatives that were picked to CAS plates, all made the normal orange halo but of 2000 other insertants which were not blue, one had a larger (approx. 10-fold) halo than the wild-type. This mutant, termed J300, was chosen for further study.

Identification of the *flu* gene defective in mutant J300

A gene bank (deLuca et al., 1998) comprising cosmids of the wide-host-range plasmid pLAFR1 and containing approximately 30 kb inserts of *R. leguminosarum* 8401pRL1JI genomic DNA was mobilized en masse into strain J300 and transconjugants picked onto CAS plates. One colony was found which had a wild-type halo. The plasmid, termed pBIO400, was isolated from this transconjugant and introduced into *E. coli* by transformation. It was then reintroduced by conjugation into the mutant J300; in all cases, the transconjugants had a wild-type appearance on CAS plates. A 16 kb EcoRI fragment, subcloned into the wide-host-range vector pRK415 to form pBIO402, also corrected the siderophore defect of J300 on CAS plates. Deleting a 1·1 kb BamHI fragment (Fig. 1) abolished the ability of pBIO402 to correct J300, indicating that this region contained the relevant genes. Various restriction fragments within and near this region were cloned into pUC18 for sequencing, and the 7395 bp sequence of this region was determined (EMBL accession number AJ007906).

At one end of the sequenced region (Fig. 1) were 430 bp of an ORF whose product was 39·5% identical to RopB, an outer-membrane protein in *R. leguminosarum* (Roest et al., 1995). Downstream of this were genes of obvious relevance to mutant strain J300 (Fig. 1). Three closely linked ORFs, all divergently transcribed from the ropB homologue, specified polypeptides that were similar to those coded by the *fluB, C* and *D* genes of *E. coli*, which are involved in the uptake of the Fe-hydroxamate complexes; FluD is the periplasmic transporter and FluB and FluC are inner-membrane proteins that transfer the Fe-siderophore complex into the cytoplasm. In *E. coli*, these three genes are in an operon *fluACDB*; the fourth gene, *fluA*, specifies the outer-membrane receptor for the Fe-ferrichrome complex (see Braun et al., 1998).

The start of *fluC* was separated by 300 bp from the ropB homologue. The FluC protein is an ATPase and the similarity in sequence of the *R. leguminosarum* FluC to other homologues was greatest at the ATP-binding site. There was also significant homology...
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FhuD, their more generally conserved C-terminal regions. An asterisk overall, the similarity to corresponding proteins in other bacteria, the similarity being greatest to the FhuC of E. coli and from the wild-type control and probing the digests with pBIO402 DNA, it was found that the TnpboA in the original mutant was in fhuB (not shown).

Identification of a pseudogene version of fhuA

Downstream of fhuB and in the opposite orientation was an unusual homologue of fhuA. In other bacteria, fhuA (or a homologue of it) encodes the outer-membrane receptor for ferric ferrihydrite. These homologues include FegA, an outer-membrane protein of B. japonicum (LeVier & Guerinot, 1996), and FptA of Pseudomonas aeruginosa (Ankenbauer & Quan, 1994). This deduced fhuA ‘pseudo’-gene product was very similar to E. coli FhuA at the C-terminal region (Fig. 2). However, there is little conservation upstream of this, and more strikingly, there are many stop codons in the R. leguminosarum DNA in all three frames. Note that in the FhuA-like proteins of other bacteria, sequence conservation is much less pronounced in their N-terminal than their C-terminal halves. Nevertheless, in this upstream region of the fhuA pseudogene ‘product’, there is significant identity (Fig. 2a) in sequence to FptA of P. aeruginosa.

The sequence suggests that this version of fhuA in R. leguminosarum is a pseudogene, an unusual feature in bacteria. Despite the limited sequence similarity to FptA in the deduced N-terminal region of R. leguminosarum FhuA, we were concerned that the abrupt break in homology of the FhuAs of E. coli and R. leguminosarum might have been artifactual, due to a rearrangement in the genome of the strain from which the bank originated or during the construction of pBIO400. To test this, DNA spanning fhuA was used to probe genomic DNA isolated from different strains of R. leguminosarum and digested with restriction enzymes that would generate diagnostic hybridizing patterns. In all cases, the hybridizing fragments corresponded to those predicted from the map of pBIO400. Thus, there was no such rearrangement nor was there another genomic copy of fhuA with a very similar sequence to the one found here (data not shown).

This pseudogene of fhuA is not expressed. A 3.3 kb EcoRI–SalI fragment whose 3’ end was in fhuA and

Fig. 1. Representation of the fhu region of R. leguminosarum. The ORFs within the sequenced region between a SalI (S) and HindIII (H) site are indicated by arrows: Orf 1 corresponds to the ropB homologue. The BamHI sites used to make the deleted form of pBIO402 are shown (B).

Fig. 2. Comparison of parts of the R. leguminosarum (Rl) FhuA pseudogene product with homologues of FhuA-like proteins in other bacteria: E. coli (Ec) FhuA, B. japonicum (Bj) FegA, P. aeruginosa (Pa) FptA. (a) Comparison of a short region of homology between R. leguminosarum FhuA and FptA towards their N-terminal ends; (b) comparisons of all four proteins in their more generally conserved C-terminal regions. An asterisk (*) indicates identity between FhuA of R. leguminosarum and all the other proteins; + indicates identity between FhuAs of E. coli and R. leguminosarum.

throughout the corresponding proteins from several bacteria, the similarity being greatest to the FhuC of E. coli (51% identity).

The proposed start of fhuD is separated by 15 bp from fhuC. Of the fhu genes identified here, the product of fhuD (the periplasmic-binding protein) has the least similarity to corresponding proteins in other bacteria, being 26% identical to FhuD of E. coli, the closest match. The proposed start of fhuB overlaps the end of fhuD, suggesting that they are translationally coupled. Overall, the R. leguminosarum FhuB protein was most similar to that of E. coli (39% identity). Compared to FhuB of other bacteria (e.g. Bacillus subtilis) the FhuB peptides of Rhizobium and E. coli are much larger, consistent with the protein having two repeated domains that span the cytoplasmic membrane (see Braun et al., 1998).

By isolating and digesting the DNA from mutant J300 and from the wild-type control and probing the digests with pBIO402 DNA, it was found that the TnpboA in the original mutant was in fhuB (not shown).
Table 2. Expression of fhuB::lacZ(pBIO403) and fhuC::lacZ(pBIO405) fusions in R. leguminosarum in high- and low-Fe cultures

<table>
<thead>
<tr>
<th>Strain</th>
<th>Succinate + Fe</th>
<th>Succinate − Fe</th>
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<tbody>
<tr>
<td>8401(pLAFR1)</td>
<td>8 ± 2</td>
<td>12 ± 3</td>
</tr>
<tr>
<td>8401(pBIO403)</td>
<td>165 ± 9</td>
<td>1245 ± 23</td>
</tr>
<tr>
<td>8401(pBIO405)</td>
<td>173 ± 8</td>
<td>1304 ± 36</td>
</tr>
</tbody>
</table>

whose 5' end included approximately 2 kb of sequence that is predicted to contain any promoter of fhuA, was cloned into the promoter-probe vector pMP220 and the resultant plasmid, pBIO410, was mobilized to R. leguminosarum. The transconjugant was grown in media with and without Fe; in both cases there was no β-galactosidase above background, showing that this copy of fhuA is not transcribed.

Expression of the fhuBCD genes

To analyse expression of fhuDCB, we mutagenized pBIO400 with the reporter transposon Tn5lacZ. One hundred mutagenized derivatives were mobilized into strain J300 and four that no longer corrected it were transformed into E. coli. Mapping showed that two were in fhuB and two in fhuC, and one insertion in each of the genes had lacZ of the Tn5lacZ in the ‘correct’ orientation. As expected, the two insertions in the ‘wrong’ orientation had undetectable levels of β-galactosidase activities when the plasmids were in R. leguminosarum. The two plasmids with lacZ in the correct orientation were termed pBIO403 (fhuB1::lacZ) and pBIO405 (fhuC1::lacZ).

Neither pBIO403 nor pBIO405 corrected the siderophore-overproduction of strain J300, showing that fhuB and C are in the same transcriptional unit, consistent with their close linkage. The two plasmids were mobilized into R. leguminosarum 8401 and the two mutations were introduced into the genome by marker exchange. For both, the homogenotes had large haloes on CAS plates.

β-Galactosidase activities were measured for the fhuC- and fhuB-fusions, both where the Tn5lacZ was plasmid-borne in pBIO403 and pBIO405 and when they had been homogenotized. The assays were done on cells grown in Fe-replete medium and in medium where Fe had been sequestered with 4,4'-dipyridyl. Levels of β-galactosidase were higher (approx. eightfold) for both fusion plasmids in the cells grown in low-Fe medium (Table 2). As expected, the levels of expression were lower with fusions in the chromosome than in the recombinant plasmids, with their higher copy number (not shown).

The decrease in fhuDCB expression in high-Fe media is similar to that found in other bacteria. Thus, the genes in Pseudomonas aeruginosa involved in production of siderophore and of cell-surface receptors are repressed by Fe via the transcriptional regulator, fur, which in many bacteria responds to Fe by regulating other genes involved in Fe metabolism (Braun et al., 1998). We had identified a fur homologue in R. leguminosarum, but fur mutants could not be isolated (deLuca et al., 1998), suggesting that, as in P. aeruginosa (Barton et al., 1996a), these are lethal. So, it is not possible to say if fhuDCB is fur-regulated. The DNA upstream of fhuC has no sequence similar to a fur box, the motif (Braun et al., 1998) to which Fur binds.

**Effects of feuQ on expression of fhu–lac fusions**

Yeoman et al. (1997) identified two genes (feuP and feuQ) which appear to be members of the ‘two-component sensor–regulator’ family. A mutation in the ‘sensor’, feuQ, caused a defect in Fe uptake though not, apparently, in siderophore synthesis or uptake. In view of the role of feuPQ in Fe acquisition, we tested if they controlled expression of fhuDCB by mobilizing pBIO403 (fhuB1::lacZ) and pBIO405 (fhuC1::lacZ) into the feuQ mutant, J100. Transconjugants were grown in Fe-replete and Fe-depleted Y medium before assaying for β-galactosidase. The feuQ mutation had no detectable effect on transcription of either fusion in the presence or absence of Fe in the media (not shown).

Strain 8401pRL1J1 and its derivatives make vicibactin as their siderophore

Dilworth et al. (1998) identified vicibactin as the sole siderophore made by a strain of R. leguminosarum bv. viciae. Using 1H NMR spectroscopy, it was shown that the identical siderophore was made by the wild-type strain 8401pRL1J1 used here. Also, the original mutant J300 and strain J302, the fhuB1::Tn5lac mutant derivative of strain 8401, also produced vicibactin, albeit in larger amounts than the wild-type (see below).

In strains 8401 and 8401pRL1J1, vicibactin production was shown to be Fe-regulated. Cells grown in MSM-20 and transferred to MSM containing various concentrations of added Fe (0–20 mM Fe) produced vicibactin in MSM-20 and MSM-10 but not MSM-20.

The fhuB mutation affects Fe-vicibactin uptake

For uptake studies, the fhuB1::Tn5lac mutant J302 and its parent 8401 were grown in MSM-0, transferred to MSM-0 or MSM-20 and harvested from MSM-0 at late exponential phase (OD_{600} 0.7–0.9) and from MSM-20 at early stationary phase (OD_{600} 2.5–3.0) to ensure the induction of siderophore synthesis. Transport of Fe complexed to citrate appeared to be constitutive and unaffected by the fhuB mutation on MSM-0 medium. For reasons that are not clear, uptake of Fe from ferric citrate by the mutant was somewhat reduced on MSM-
Table 3. Rates of $^{55}$Fe transport for *R. leguminosarum* strains 8401 and J302 grown in MSM-0 and MSM-20

Rates are expressed as pmol Fe min$^{-1}$ (mg protein)$^{-1}$. ND, Not detectable.

| $^{55}$Fe complexed with Cells grown in MSM-0 Cells grown in MSM-20 |
|------------------|------------------|
| Vicibactin*      | 52 ± 12          | ND |
| Citrate*         | 100 ± 9          | 83 ± 25 |
| Vicibactin concn | 120              | 66 ± 10 |
| supernatant (µM)$^+$| 25              | 230 |

* Values are the means of four determinations ± standard deviation.
† Values are the means of two determinations.

20 medium (Table 3). Similar results were found with citrate uptake in the original mutant J300 in comparison to its parental strain 8401pRL1JI (not shown).

Transport of $^{55}$Fe complexed to vicibactin into cells of strain 8401 grown in MSM-0 was linear over time (5 min), but when grown in MSM-20, cells did not detectably accumulate $^{55}$Fe (Table 3). In contrast, uptake of Fe by cells of strain J302 grown in MSM-0 was markedly lower compared to strain 8401. Thus the *fbu* mutation causes an approximately 80% reduction in the uptake of the Fe-vicibactin complex. Interestingly, though, there is still residual uptake activity for the complex, suggesting that there may be an alternative, less efficient mechanism involved in vicibactin uptake. Under conditions of high external Fe (MSM-20), this residual uptake is no longer seen, indicating that higher levels of Fe repress its activity. However, at high Fe levels, the mutant J302 still produces vicibactin into the medium, unlike the wild-type, suggesting that vicibactin synthesis, but not uptake, is not totally repressed by exposure of cells to high Fe conditions (Table 3). This could be due to the mutant still being deprived of Fe even when the external Fe concentration was relatively high.

Table 4. Total Fe concentrations of *R. leguminosarum* strains 8401 and J302 grown in MSM-0 and MSM-20

<table>
<thead>
<tr>
<th>Strain</th>
<th>Fe concn [µg (g dry wt)$^{-1}$]</th>
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<tbody>
<tr>
<td></td>
<td>Cells grown in Cells grown in</td>
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<tr>
<td></td>
<td>MSM-0</td>
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<td>8401</td>
<td>105</td>
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<tr>
<td>J302</td>
<td>71</td>
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<tr>
<td>WSM710</td>
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</table>

Total Fe concentrations in strains 8401 and J302

Fe concentrations were measured in cells of 8401 and J302 grown in MSM-0 and MSM-20; data for *R. leguminosarum* biovar *vicieae* WSM710 have been added for comparison (Table 4). In J302, the Fe levels in cells grown in MSM-0 or MSM-20 were approximately 70% of those for its parent 8401. The critical internal Fe concentration for repression of vicibactin synthesis in strain 8401 therefore appears to be between 105 and 178 µg (g dry weight)$^{-1}$; it is likely to be nearer the upper figure since cells grown in MSM-0 before transfer to MSM-20 derepress vicibactin synthesis in late stationary phase.

Effects of *fbu* mutations on symbiotic N$_2$ fixation

Wild-type *R. leguminosarum* bv. *vicieae* 8401pRL1JI, the original *fbu* mutant J300 and homogenotized derivatives of 8401pRL1JI carrying the *fbu*1::Tn5lac and the *fbu*1::Tn5lac mutations were used to inoculate peas. In terms of the time of onset of nodulation, the numbers and sizes of nodules formed 21 d post-inoculation and the levels of N$_2$ fixation, as measured by $\text{C}_2\text{H}_4$ reduction, the *fbu* mutants were indistinguishable from wild-type. Bacteria isolated from the nodules induced by the *fbu* mutants all made large haloes on CAS plates.

The finding that *fbu* mutants appear to obtain sufficient Fe for the purposes of N$_2$ fixation is consistent with other observations that the capacity to make siderophores *in vitro* may not be required by bacteroids (Reigh & O’Coanell, 1993; Fabiano et al., 1995). It is not clear if this is because there is a bacteroid-specific siderophore system or that bacteroids receive their Fe in the ferrous form, via the action of a ferri-reductase in the plant (LeVier et al., 1996), or whether bacteroids gain Fe as ferric citrate which can cross the peribacteroid membrane (LeVier et al., 1996).

Conclusions

We identified three genes, *fbuB*, *C* and *D*, and a form of *fbuA* in a strain of *R. leguminosarum*, with homologues in other bacteria. Upstream of *fbuC*, and in the opposite orientation, is a homologue of *ropB*, a gene which encodes an outer-membrane protein in *R. leguminosarum* and Brucella melitensis but which has not been implicated in Fe uptake (Roest et al., 1995; Vizcaino et al., 1996). From complementation tests and
their close proximity, \( fhuB, C \) and \( D \) appear to be co-transcribed. The \( fhuB \) gene is functional since mutations in it markedly lower uptake of \(^{58}\text{Fe} \) complexed to vicibactin. Expression of \( fhuDCB \) is subject to modulation by Fe availability. The reason for the phenotype of a large halo on CAS plates of \( fhuB \) and \( fhuC \) mutants is presumably that vicibactin produced by these strains is not internalized and so accumulates in the medium. Further, the defect in vicibactin uptake would lead to an intracellular Fe deficit, resulting in the \( fhu \) genes being expressed at higher levels. Note that starvation is not severe, as indicated by the substantial levels of intracellular Fe in the mutant. Since \( fhuB \) mutations specifically affect vicibactin-mediated but not citrate-based Fe uptake, \( R. \) leguminosarum, like other bacteria, must have a dedicated hydroxamate uptake system. Carson et al. (1994) have indeed suggested at least three systems for trihydroxamate-chelated Fe transport in \( R. \) leguminosarum biovar \( viciae \) WSM710, which produces the same siderophore as 8401 and 8401pRL1J1.

It is striking that in \( R. \) leguminosarum there is a version of \( fhuA \) which is close to \( fhuDCB \), but in a separate transcriptional unit. Sequencing upstream of \( fhuA \) showed that it is not in a larger operon (not shown). The sequence of this \( fhuA \) indicated that it is a pseudogene with many stop codons and that it is not transcribed. Pseudogenes are unusual in bacteria. In \( \text{Actinobacillus pleuropneumoniae} \), there is a defective homologue of \( hlyB \), involved in haemolysin production in other bacteria (Chang et al., 1991). In \( \text{Bordetella pertussis} \) a gene (\( \text{fimA} \)) with homology to genes involved in synthesis of fibrin has been identified (Willems et al., 1992) and in \( \text{Borrelia hermsii} \) there is a pseudogene for an antigenic protein (Restrepo et al., 1994). These cases involve cell surface proteins, just as \( \text{FhuA} \), described here, does. In \( \text{E. coli} \), \( \text{FhuA} \) can act as a receptor for different bacteriophages, colicins and antibiotics and \( fhuA \) mutants can confer resistance to one or more of such agents (see Killman & Braun, 1992; Killman et al., 1995). If the functional precursor of the \( \text{FhuA} \) protein identified here was a target for a rhizobiophage or rhizobacin, there would be strong selection pressure to remove this vulnerable feature if the strain had another, functional equivalent of \( \text{FhuA} \). We have not yet found such a functional copy of \( \text{FhuA} \) in \( R. \) leguminosarum, though in \( \text{B. japonicum} \), such a version of \( \text{FhuA} \) has been identified (LeVier & Guerinot, 1996).

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**REFERENCES**


flu genes of Rhizobium


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