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

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A mutant of \textit{Rhizobium leguminosarum} was isolated which fails to take up the siderophore vicibactin. The mutation is in a homologue of \textit{fhuB}, which in \textit{Escherichia coli} specifies an inner-membrane protein of the ferric hydroxamate uptake system. In \textit{Rhizobium}, \textit{fhuB} is in an operon \textit{fhuDCB}, which specifies the cytoplasmic membrane and periplasmic proteins involved in siderophore uptake. \textit{fhuDCB} mutants make vicibactin when grown in Fe concentrations that inhibit its production in the wild-type. Nodules on peas induced by \textit{fhuDCB} mutants were apparently normal in N\textsubscript{2} fixation. Transcription of an \textit{fhuDCB-lacZ} fusion was Fe-regulated, being approximately 10-fold higher in Fe-depleted cells. Downstream of \textit{fhuB}, in the opposite orientation, is a version of \textit{fhuA} whose homologues in other bacteria specify hydroxamate outer-membrane receptors. This \textit{fhuA} gene appears to be a pseudogene with stop codons and undetectable expression.

**Keywords**: \textit{fhu} genes, iron-mediated regulation, pseudogene, \textit{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 Fe\textsuperscript{3+} form, it is very insoluble at normal pH and pO\textsubscript{2}. Therefore, many bacteria make and export low-M\textsubscript{r} compounds, known collectively as siderophores, which bind avidly to Fe\textsuperscript{3+}. 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 N\textsubscript{2}-fixing bacteria since it is a component of nitrogenase and electron donors specifically involved in N\textsubscript{2} reduction. Also, in the case of the symbiosis between legumes and bacteria known collectively as the rhizobia, the bacteria in N\textsubscript{2}-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 (Riou 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 \textit{Sinorhizobium} (formerly \textit{Rhizobium}) \textit{meliloti} which fail to make rhizobactin (Barton et al., 1996b) or anthranilate (Barsomian et al., 1992) are defective in N\textsubscript{2} fixation on alfalfa, but others are unaffected (Fabiano et al., 1995; Reigh & O'Connell, 1993).

A \textit{Rhizobium leguminosarum} mutant defective in siderophore synthesis (Yeoman et al., 1997) failed to fix N\textsubscript{2} in pea nodules, and was also defective in synthesizing all c-type cytochromes. The mutation was in the \textit{cycHJKL} operon, which in \textit{R. leguminosarum}, \textit{S.}
J. B. STEVENS and OTHERS

respiratory defect that causes the Fix- phenotype maturation of c-type cytochromes; it is, in fact, the meliloti 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-N6-hydroxy-2-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 MSO; thus MSM-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 MSM-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(CIO4)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

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Table 1. Strains and plasmids used in this study

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Characteristics</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Strains</td>
<td>R. leguminosarum bv. viciae; str</td>
<td>Downie et al. (1983)</td>
</tr>
<tr>
<td>8401pRL1JI</td>
<td>R. leguminosarum bv. viciae; str</td>
<td>Lamb et al. (1982)</td>
</tr>
<tr>
<td>8401</td>
<td>8401pRL1JI cured of sym plasmid pRL1JI</td>
<td>Yeoman et al. (1997)</td>
</tr>
<tr>
<td>J100</td>
<td>8401pRL1JI feuQ::TnSlac</td>
<td>This work</td>
</tr>
<tr>
<td>J300</td>
<td>8401pRL1JI, fluB1::TnphoA</td>
<td>This work</td>
</tr>
<tr>
<td>J302</td>
<td>8401 fluB1::TnSlac</td>
<td>Wood (1966)</td>
</tr>
<tr>
<td>803</td>
<td>E. coli, used for transformation</td>
<td>Simon et al. (1989)</td>
</tr>
<tr>
<td>A118</td>
<td>E. coli with TnSlacZ</td>
<td></td>
</tr>
<tr>
<td>Plasmids</td>
<td>Vector for R. leguminosarum gene bank</td>
<td>Friedman et al. (1982)</td>
</tr>
<tr>
<td>pLAFR1</td>
<td>Wide-host-range cloning vector; Tet'</td>
<td>Keen et al. (1988)</td>
</tr>
<tr>
<td>pRK415</td>
<td>Helper plasmid</td>
<td>Figurski &amp; Helinski (1979)</td>
</tr>
<tr>
<td>pRK2013</td>
<td>Incompatible plasmid used for marker exchange</td>
<td>Beringer et al. (1978)</td>
</tr>
<tr>
<td>pPH111</td>
<td>Used for delivering TnphoA</td>
<td>Taylor et al. (1989)</td>
</tr>
<tr>
<td>pRT733</td>
<td>Used for sequencing</td>
<td>Messing et al. (1983)</td>
</tr>
<tr>
<td>pUC18</td>
<td>Freeze-dried</td>
<td>Spank et al. (1985)</td>
</tr>
<tr>
<td>pMP220</td>
<td>Promoter-probe vector</td>
<td></td>
</tr>
<tr>
<td>pBIO400</td>
<td>16 kb EcoRI fragment from pBIO400 cloned in pRK415</td>
<td></td>
</tr>
<tr>
<td>pBIO403</td>
<td>pBIO400 fluB1::TnSlac</td>
<td>This work</td>
</tr>
<tr>
<td>pBIO405</td>
<td>pBIO400 fluCl::TnSlac</td>
<td>This work</td>
</tr>
</tbody>
</table>
The study focused on the genetic manipulation of Rhizobium strains to understand the mechanism of siderophore secretion. The authors isolated mutants altered in siderophore secretion or uptake, and these mutants were characterized by their ability to form normal orange halos on CAS plates. The plasmid pLAFR1 was used as a helper in tri-parental mating to transfer the plasmids to E. coli. The DNA was isolated from the mutants and sequenced to identify the gene(s) involved in siderophore secretion.

**RESULTS AND DISCUSSION**

**Isolation of siderophore-overproducing Rhizobium mutant**

In a search for mutants altered in siderophore secretion or uptake, *R. leguminosarum* bv. *viciae* 8401pRL1J1 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 inserts 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 *fhu* 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* 8401pRL1J1 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 *fhuB*, *C* and *D* genes of *E. coli*, which are involved in the uptake of the Fe-hydroxamate complexes; FhuD is the periplasmic transporter and FhuB and FhuC are inner-membrane proteins that transfer the Fe-siderophore complex into the cytoplasm. In *E. coli*, these three genes are in an operon *fhuACDB*, where the fourth gene, *fhuA*, specifies the outer-membrane receptor for the Fe-ferrichrome complex (see Braun et al., 1998).

The start of *fhuC* was separated by 300 bp from the *ropB* homologue. The FhuC protein is an ATPase and the similarity in sequence of the *R. leguminosarum* FhuC to other homologues was greatest at the ATP-binding site. There was also significant homology...
J. B. STEVENS and OTHERS

Comparison of parts of the \textit{R. leguminosarum} (RI) FhuA pseudogene product with homologues of FhuA-like proteins in other bacteria: \textit{E. coli} (Ec) FhuA, \textit{B. japonicum} (Bj) FegA, \textit{P. aeruginosa} (Pa) FptA. (a) Comparison of a short region of homology between \textit{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 \textit{R. leguminosarum} and the other proteins; + indicates identity between FhuAs of \textit{E. coli} and \textit{R. leguminosarum}.

Identification of a pseudogene version of \textit{fhuA}

Downstream of \textit{fhuB} and in the opposite orientation was an unusual homologue of \textit{fhuA}. In other bacteria, \textit{fhuA} (or a homologue of it) encodes the outer-membrane receptor for ferric ferriochrome. These homologues include FegA, an outer-membrane protein of \textit{B. japonicum} (LeVier & Guerinot, 1996), and FptA of \textit{Pseudomonas aeruginosa} (Ankenbauer & Quan, 1994). This deduced \textit{fhuA} ‘pseudo’-gene product was very similar to \textit{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 \textit{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 \textit{fhuA} pseudogene ‘product’, there is significant identity (Fig. 2a) in sequence to FptA of \textit{P. aeruginosa}.

The sequence suggests that this version of \textit{fhuA} in \textit{R. leguminosarum} is a pseudogene, an unusual feature in bacteria. Despite the limited sequence similarity to FptA in the deduced N-terminal region of \textit{R. leguminosarum} FhuA, we were concerned that the abrupt break in homology of the FhuAs of \textit{E. coli} and \textit{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 \textit{fhuA} was used to probe genomic DNA isolated from different strains of \textit{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 \textit{fhuA} with a very similar sequence to the one found here (data not shown).

This pseudogene of \textit{fhuA} is not expressed. A 3-3 kb EcoRI–SalI fragment whose 3’ end was in \textit{fhuA} and

FhuB of other bacteria (e.g. \textit{Bacillus subtilis}) the FhuB peptides of \textit{Rhizobium} and \textit{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 TnphoA in the original mutant was in \textit{fhuB} (not shown).

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

The proposed start of \textit{fhuD} is separated by 15 bp from \textit{fhuC}. Of the \textit{fhu} genes identified here, the product of \textit{fhuD} (the periplasmic-binding protein) has the least similarity to corresponding proteins in other bacteria, being 26% identical to FhuD of \textit{E. coli}, the closest match. The proposed start of \textit{fhuB} overlaps the end of \textit{fhuD}, suggesting that they are translationally coupled. Overall, the \textit{R. leguminosarum} FhuB protein was most similar to that of \textit{E. coli} (39% identity). Compared to

Fig. 1. Representation of the \textit{fhu} region of \textit{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).
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 \etal, 1998). We had identified a \( fur \) homologue in \( R. \) \( leguminosarum \), but \( fur \) mutants could not be isolated (deLuca \etal, 1998), suggesting that, as in \( P. \) \( aeruginosa \) (Barton \etal, 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 \etal, 1998) to which \( Fur \) binds.

**Effects of \( feuQ \) on expression of \( fhu\)–\( lac \) fusions**

Yeoman \etal (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 \( feuP \) in Fe acquisition, we tested if they controlled expression of \( fhuDCB \) by mobilizing \( pBI0403 \) (\( fhuB1\)-\( lacZ \)) and \( pBI0405 \) (\( fiuC1\)-\( lacZ \)) into the \( feuQ \) mutant, J100. Transconjugants were grown in Fe-replete and Fe-depleted Y medium before assaying for \( \beta \)-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 \etal (1998) identified vicibactin as the sole siderophore made by a strain of \( R. \) \( leguminosarum \) \( bv. \) \( viciae \). Using \( \textsuperscript{1}H \) 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 \( fhuB1::\text{Tn5lac} \) 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-0 and MSM-10 but not MSM-20.

**The \( fhuB \) mutation affects Fe-vicibactin uptake**

For uptake studies, the \( fhuB1::\text{Tn5lac} \) 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 (\( \text{OD}_{600} \) 0.7–0.9) and from MSM-20 at early stationary phase (\( \text{OD}_{600} \) 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-

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**Table 2. Expression of \( fhuB::lacZ(pBI0403) \) and \( fhuC::lacZ(pBI0405) \) fusions in \( R. \) \( leguminosarum \) in high- and low-Fe cultures**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Succinate + Fe</th>
<th>Succinate – Fe</th>
</tr>
</thead>
<tbody>
<tr>
<td>8401(pLAFR1)</td>
<td>8 ± 2</td>
<td>12 ± 3</td>
</tr>
<tr>
<td>8401(pBI0403)</td>
<td>165 ± 9</td>
<td>1245 ± 23</td>
</tr>
<tr>
<td>8401(pBI0405)</td>
<td>173 ± 8</td>
<td>1304 ± 36</td>
</tr>
</tbody>
</table>

\( pLAFR1 \) is the vector plasmid control. \( \beta \)-Galactosidase activities are given in Miller units. The values tabulated are means ± standard errors, obtained from four independently grown cultures.

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 \( \beta \)-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 \( \text{Tn5lacZ} \). 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 \( \text{Tn5lacZ} \) in the ‘correct’ orientation. As expected, the two insertions in the ‘wrong’ orientation had undetectable levels of \( \beta \)-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.

\( \beta \)-Galactosidase activities were measured for the \( fhuC \)- and \( fhuB \)-fusions, both where the \( \text{Tn5lacZ} \) 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 \( \beta \)-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).
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.

<table>
<thead>
<tr>
<th>$^{55}$Fe complexed with</th>
<th>Cells grown in MSM-0</th>
<th>Cells grown in MSM-20</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>8401</td>
<td>J302</td>
</tr>
<tr>
<td>Vicibactin*</td>
<td>52 ± 12</td>
<td>10 ± 7</td>
</tr>
<tr>
<td>Citrate*</td>
<td>100 ± 9</td>
<td>96 ± 14</td>
</tr>
<tr>
<td>Vicibactin concn in supernatant (µM)†</td>
<td>120</td>
<td>160</td>
</tr>
</tbody>
</table>

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

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>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cells grown in MSM-0</td>
</tr>
<tr>
<td>8401</td>
<td>105</td>
</tr>
<tr>
<td>J302</td>
<td>71</td>
</tr>
<tr>
<td>WSM710</td>
<td>68</td>
</tr>
</tbody>
</table>

20 medium (Table 3). Similar results were found with citrate uptake in the original mutant J300 in comparison to its parental strain 8401pRL1JJ (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 *fhuB* 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.

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 *fhu* mutations on symbiotic N$_2$ fixation

Wild-type *R. leguminosarum* bv. *vicieae* 8401pRL1JJ, the original *fhuB* mutant J300 and homogenotized derivatives of 8401pRL1JJ carrying the *fhuB*::Tn5lac and the *fhuCl*::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 C$_2$H$_4$ reduction, the *fhu* mutants were indistinguishable from wild-type. Bacteria isolated from the nodules induced by the *fhu* mutants all made large haloes on CAS plates.

The finding that *fhu* 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'Coenell, 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, *fhuB*, *C* and *D*, and a form of *fhuA* in a strain of *R. leguminosarum*, with homologues in other bacteria. Upstream of *fhuC*, 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 $^{59}$Fe complexed to vicinibactin. 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 vicinibactin produced by these strains is not internalized and so accumulates in the medium. Further, the defect in vicinibactin 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 vicinibactin-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 *Actinobacillus pleuropneumoniae*, there is a defective homologue of *hlyB*, involved in haemolysin production in other bacteria (Chang *et al.*, 1991). In *Bordetella pertussis* a gene (*fimA*) with homology to genes involved in synthesis of fibrin has been identified (Willems *et al.*, 1992) and in *Borrelia hermsii* there is a pseudogene for an antigenic protein (Restrepo *et al.*, 1994). These cases involve cell surface proteins, just as FhuA, described here, does. In *E. coli*, 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 FhuA protein identified here was a target for a rhizobiophage or rhizobcin, there would be strong selection pressure to remove this vulnerable feature if the strain had another, functional equivalent of FhuA. We have not yet found such a functional copy of FhuA in *R. leguminosarum*, though in *B. japonicum*, such a version of FhuA has been identified (LeVier & Guerinot, 1996).

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**REFERENCES**


*fbu* genes of *Rhizobium*


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