Chapter 3

Cloning and expression of E. coli fhuA gene: Effect of ferrichrome utilizing ability on the growth, nodulation and rhizospheric survivability in the C. cajan rhizobial isolates under lab conditions.

The ways of the bacteria are odd.
They are understood only by God
Any man who proclaims that he fathomed their aims,
Is, either a fool or a fraud
- I. D. Dubvır
3.1 INTRODUCTION
To meet iron requirements for growth, most microorganisms have developed high affinity iron uptake systems for scavenging and transporting ferric iron (Braun and Killmann, 1999). These involve low molecular weight compounds called siderophores which bind ferric iron with high affinity (Crosa and Walsh, 2002; Neilands, 1995) and several membrane bound and periplasmic proteins that function together in the uptake of the ferric-siderophore complex (Faraldo-Gómez and Sansom, 2003). This system comprises of, an outer membrane receptor imparting specificity for uptake and which function as gated porin channels working in concert with TonB protein that energizes the receptor protein and helps in internalization of Fe$^{3+}$-siderophore complex (Braun and Killmann 1999; Rattlede and Dover 2001). More than 500 distinct siderophores have been reported to be secreted by microorganisms and their iron ligation groups have been classified into three main chemical types: hydroxamate, catecholate and hydroxyacid (Wandersman and Delepelaire, 2004).

Rhizobia are a group of gram-negative bacilli possessing the ability to form a nitrogen-fixing symbiosis with members of the *Leguminaceae* family of plants. Recent taxonomy recognizes major groups of rhizobia as belonging to the *Mesorhizobium-Sinorhizobium-Rhizobium* group, the *Bradyrhizobium* group, the *Azorhizobium* group, *Methylobacterium* group, and *Burkholderia* group (Sahgal and Johri 2003; Zakhia and deLajudi, 2001). While many rhizobial strains have the potential to increase plant growth and yield, poor nodulation efficiencies due to low survivability as free-living soil microorganisms are a problem and iron limitation is one of the important factors contributing to their low survivability (O'Hara et al., 1988a; Hemantaranjan and Garg, 1986). When applied to the soil as biofertilizers, rhizobia have to face additional challenges to enter into a nitrogen fixing symbiosis. In the soil, they have to survive and increase their numbers for attaining high frequency of nodulation. As endosymbionts, they must have efficient mechanisms to acquire iron from the host plant as their iron requirement is high, producing three times more cytochromes as compared to when they are free living (Sangwan and O'Brian, 1992) and synthesizing nitrogenase (containing at least 30 iron atoms), which is 10-12 % of the total bacterial protein (Verma and Long, 1983).
Fluorescent pseudomonads are known to efficiently colonize various ecological niches, which is largely attributed to the diverse and sophisticated iron uptake systems they possess. They have an ability to utilize a large number of heterologous siderophores via different TonB dependent siderophore receptors. Studies report 32 putative siderophore receptors in *Pseudomonas aeruginosa* (Dean and Poole 1993; Ankenbauer and Quan 1994), 29 in *Pseudomonas putida*, 27 in *Pseudomonas fluorescens* and 23 in *Pseudomonas syringae* (Cornelis and Matthijs, 2002).

Diverse types of siderophores are produced by the different rhizobial genera. For instance, *Rhizobium leguminosarum* bv. *viciae* and *Sinorhizobium meliloti* secrete hydroxamate siderophores (Persmark et al., 1993; Carson et al. 2000). Citrate is the siderophore secreted by *Bradyrhizobium japonicum* (Guerinot et al., 1990). Several uncharacterized catecholate siderophores are produced by rhizobia of chick pea (Roy et al., 1994), cowpea (Modi et al., 1985; Jadhav and Desai, 1992) and pigeon pea (*C. cajan*) (Khan et al., 2006).

In addition to being able to use their own ferri-siderophore complexes, *Sinorhizobium meliloti* and *Bradyrhizobium japonicum* can also utilize iron complexed to siderophores made by other species (Smith and Neilands, 1984; Plessner et al., 1993). *Bradyrhizobium japonicum* 61A152 produces citrate (Guerinot et al., 1990) but can utilize a variety of other siderophores produced by soil microflora like ferrichrome and rhodotorulic acid, produced by fungi making it more competitive in the rhizosphere (Plessner et al., 1993). Most notable among the heterologous siderophores utilized by these rhizobia is ferrichrome, a prototypical hydroxamate type siderophore secreted by several fungi such as *Ustilago* spp (Payne, 1994). Ferrichrome, which is produced by many soil fungi (Szaniszlo et al., 1981, Winklemann, 1986), is present in the soil in high concentrations (Powell et al., 1983). The hydroxamate type siderophore concentration in soil is reported to be as high as 10µM and ferrichrome amongst these, constitutes a major fraction (Crowley et al., 1987). Ferrichrome is produced by a variety of soil fungi, and is therefore the most likely iron source in the rhizosphere. As reported by Jurkevitch and associates (1992), majority of rhizospheric organisms are ferrichrome utilizers. Possession of ferrichrome utilization ability by any soil organism will therefore have positive implications on its survival. But most of the rhizobial
isolates and other nodule bacteria tested in our laboratory failed to utilize hydroxamate type of siderophores including ferrichrome (Joshi et al., 2006; Khan et al., 2006).

The uptake system for $\text{Fe}^{3+}$-ferrichrome known as the $\text{fhu}$ system in $\text{E. coli}$ (Braun, 1995), consists of $\text{fhuACDB}$ operon, of which $\text{fhuA}$ encodes the multifunctional outer membrane protein (78 kDa) that acts not only as the ferrichrome-iron receptor but also as the receptor for several phages, for the bacterial toxin colicin M and for antibiotics such as albomycin and microcin J25 (Ferguson et al., 2001).

Rhizobia shown to either produce or utilize hydroxamate types of siderophores have been found to possess genes similar to the $\text{fhu}$ system of $\text{E. coli}$ e.g. $\text{R. leguminosarum}$ and $\text{B. japonicum}$ have been shown to possess $\text{fhuA}$ orthologs (LeVier et al., 1996; Yeoman et al; 2000) of which the $\text{B. japonicum}$ gene $\text{fegA}$ has been demonstrated to be responsible for the uptake of iron-ferrichrome complex (Benson et al., 2005). $\text{FegA}$ encodes a protein with significant amino acid similarity to a well characterized ferrichrome receptor $\text{fhuA}$ from $\text{Escherichia coli}$ (Coulton et al., 1986) and $\text{Vibrio cholerae}$ (Rogers et al., 2000). $\text{S. meliloti}$ also has two $\text{fhuA}$ homologues in its genome (Lynch et al., 2001) although no functional studies regarding them are available. Not much is however known about the hydroxamate uptake system in other rhizobia, especially those that synthesize catecholate types of siderophores.

In the earlier work (previous chapter) we have shown that majority of nodule isolates from $\text{C. cajan}$ produce catecholate siderophores and that they are more proficient at utilization of heterologous catecholates rather than hydroxamates (Khan et al., 2006). The isolates having the ability to utilize wide variety of siderophores showed growth stimulation under iron limited conditions in presence of exogenous siderophores as well as under co-inoculated conditions. Similar type of environment is present in soil where the rhizobial inoculant has to compete with the other soil microflora for siderophores in order to have a better survivability. Since soil is rich in hydroxamate type siderophores than catecholates and that too in ferrichrome, which most of the rhizobial cultures isolated by us from $\text{C. cajan}$ were unable to utilize, it was speculated that if these cultures were engineered to use ferrichrome
a prototype of hydroxamate type siderophore, then this might confer upon these isolates with an increased and better survivability under laboratory as well as in soil conditions. Thus in the present study we have dealt with the cloning of $fhuA$ from *E. coli* and its expression in rhizobial isolate IC3123 (used as a bioinoculant for *C. cajan* procured from IARI, New Delhi) and ST1, a nodule isolate from pigeon pea plant isolated in our lab. Both failed to utilize ferri-ferrichrome as well as iron bound to other hydroxamate siderophores. Expression of the *E. coli fhuA* gene enabled these bacterial strains to utilize ferri-ferrichrome as iron source and conferred upon them a better survival in presence of this siderophore. The $fhuA$ transformants of IC3123 and ST1 were tested *in planta* to determine the relative importance of siderophore utilization in symbiotic and soil environments.

### 3.2 MATERIAL AND METHOD

#### 3.2.1 Bacterial strains used in the study:

The bacterial isolates and plasmids used in this study along with their genotype and properties are tabulated in Table 3.1. *a* Ap, Cm, are ampicillin, chloramphenicol, trimethoprim and streptomycin resistance, respectively; *mob*, mobilization. *b* Rhizobial isolates used for transformation with pGR1
Table 3.1: Strains used in this study

<table>
<thead>
<tr>
<th>Bacterial/Fungal strains or plasmids</th>
<th>Properties</th>
<th>Reference or source</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>E. coli</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DH5α</td>
<td>Used for plasmid transformation</td>
<td>Sambrook and Russell, 2001</td>
</tr>
<tr>
<td>S17-1</td>
<td>Used for conjugal transfer of pGR1 into rhizobia</td>
<td>Simon et al., 1983</td>
</tr>
<tr>
<td>BL21(DE3)::pET17b fhuA</td>
<td>Used as source of the <em>fhuA</em> gene</td>
<td>Gifted by Dr. Ranjan</td>
</tr>
<tr>
<td>MB97</td>
<td>AB2847 Δ <em>fhuA</em></td>
<td>Braun, 2003</td>
</tr>
<tr>
<td>MB97::pGR1</td>
<td>MB97 carrying <em>fhuA</em> gene in pUCPM18</td>
<td>This study</td>
</tr>
<tr>
<td><strong>Rhizobial isolates</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IC3109</td>
<td>Wild type, bioinoculant used for <em>C. cajan</em></td>
<td>IARI, New Delhi, India</td>
</tr>
<tr>
<td>IC3123</td>
<td>Wild type &lt;sup&gt;b&lt;/sup&gt; bioinoculant used for <em>C. cajan</em></td>
<td>IARI, New Delhi, India</td>
</tr>
<tr>
<td>IC3163</td>
<td>Wild type bioinoculant used for <em>C. cajan</em></td>
<td>IARI, New Delhi, India</td>
</tr>
<tr>
<td>IC3169</td>
<td>Wild type bioinoculant used for <em>C. cajan</em></td>
<td>IARI, New Delhi, India</td>
</tr>
<tr>
<td>ST1</td>
<td>Wild type &lt;sup&gt;b&lt;/sup&gt;</td>
<td>Lab. isolate</td>
</tr>
<tr>
<td>IC3123::pGR1</td>
<td>IC3123 carrying <em>fhuA</em> gene in pUCPM18</td>
<td>This study</td>
</tr>
<tr>
<td>ST1::pGR1</td>
<td>ST1 carrying <em>fhuA</em> gene in pUCPM18</td>
<td>This study</td>
</tr>
<tr>
<td><strong>Pseudomonas strains</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>P. putida</em> KT2440</td>
<td>Lab. collection</td>
<td></td>
</tr>
<tr>
<td><em>P. fluorescens</em></td>
<td>Lab. collection</td>
<td></td>
</tr>
<tr>
<td>ATCC13525</td>
<td>Used as a source of heterologous siderophores</td>
<td>MTCC, Chandigarh, India</td>
</tr>
<tr>
<td><strong>Pseudomonas aeruginosa</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MTCC2453</td>
<td>Used as a source of ferrichrome</td>
<td>MTCC, Chandigarh, India</td>
</tr>
<tr>
<td><strong>Ustilago maydis</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1474</td>
<td>Used as a source of rhodotorulic acid</td>
<td>MTCC, Chandigarh, India</td>
</tr>
<tr>
<td><strong>Rhodotorula mucilaginosa</strong> 850</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Plasmids</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pUCPM18</td>
<td>pUCP19 with <em>mob</em> fragment, Ap&lt;sup&gt;+&lt;/sup&gt;</td>
<td>Hester et al., 2000</td>
</tr>
<tr>
<td>pTO4</td>
<td>pBR322 cma cmi, Ap&lt;sup&gt;+&lt;/sup&gt;</td>
<td>Ölschläger et al., 1984</td>
</tr>
<tr>
<td>pTUC203</td>
<td>pACYC184 mejABCD, Cm&lt;sup&gt;+&lt;/sup&gt;</td>
<td>Solbiati et al., 1996</td>
</tr>
<tr>
<td>pGR1</td>
<td>pUCPM18 with <em>fhuA</em>, Ap&lt;sup&gt;+&lt;/sup&gt;</td>
<td>This study</td>
</tr>
</tbody>
</table>

Construction of a transgenic *Rhizobium* for *Cajanus cajan* (pigeon pea)

*By cloning and expression of a genomic region encoding iron siderophore receptor*
3.2.2 DNA Manipulation: (Sambrook and Russell, 2001)

3.2.2.1 Genomic DNA isolation from the bacteria was done by the method of Ausubel et al (1992).

3.2.2.2 Plasmid isolation: All the plasmid isolations were performed by alkaline lysis method as per Sambrook and Russell, 2001.

3.2.2.3 Agarose gel electrophoresis:
An aliquot of the plasmid extracted (3 μl) was analysed on a 1.0% agarose gel (Sambrook and Russell, 2001) containing ethidium bromide (0.5 μg/ml). The gels were visualized under UV light in a transilluminator and photographed subsequently.

3.2.2.4 Identification of the rhizobial strains:
A fragment corresponding to 1100bp of the 16S rRNA gene was amplified using the universal eubacterial primers 5’GAGAGTTTGATCCTGGCTCAG 3’ (forward primer) and 5’GCTCGTTGCGGGACTTAACC3’ (reverse primer). Approximately 800bp sequence information of the amplified fragment was obtained through the sequencing service provided by Bangalore Genei Pvt Ltd, India. The sequence data were matched using tools provided at Ribosomal Database Project (RDP) II.

3.2.2.5 Restriction Endonuclease (RE) Digestion:
The vector pET17b containing the fhuA gene was double digested with BglIII and HindIII

Reaction system:

<table>
<thead>
<tr>
<th>Components</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA (100μg/μl)</td>
<td>10.0 μl</td>
</tr>
<tr>
<td>Buffer C (10X)</td>
<td>5.0μl</td>
</tr>
<tr>
<td>Enzyme BglIII (10U/μl)</td>
<td>1.5μl</td>
</tr>
<tr>
<td>Enzyme HindIII (20U/μl)</td>
<td>0.5μl</td>
</tr>
<tr>
<td>Autoclaved D/W</td>
<td>33.0μl</td>
</tr>
<tr>
<td>Total</td>
<td>50.0 μl</td>
</tr>
</tbody>
</table>
The reaction mixture was incubated overnight at 37°C.

**NOTE:** If a double digestion is performed in one single system then it is essential to look for a compatible buffer. *HindIII* shows 100% activity in Buffer C and 25% in Buffer B of Bangalore Genei Pvt India. *BgIII* shows 100% activity in Buffer B and 75% in Buffer C. Therefore Buffer C was chosen for the double digestion.

Vector pUCPM18 into which the *fhuA* had to be sub-cloned was first digested with *HindIII* and then subsequently digested with *BamHI*.

**Reaction system:**

<table>
<thead>
<tr>
<th>Components</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA (100µg/µl)</td>
<td>10.0 µl</td>
</tr>
<tr>
<td>Buffer C (10X)</td>
<td>5.0 µl</td>
</tr>
<tr>
<td>Enzyme <em>HindIII</em> (20U/µl)</td>
<td>0.5 µl</td>
</tr>
<tr>
<td>Autoclaved D/W</td>
<td>33.5 µl</td>
</tr>
<tr>
<td>Total</td>
<td>50.0 µl</td>
</tr>
</tbody>
</table>

The reaction mixture was incubated overnight at 37°C.

**NOTE:** When a sequential double digestion is performed and the RE’s do not have any compatible buffer then it is important to digest first with the RE whose buffer has minimum salt concentration and then use the second RE with higher salt concentration. If this is taken care of then probably the second digestion may not work well.

After the first digestion 0.5µl of *BamHI* (10U/µl) and 0.5µl of 100X BSA was added into the same system. Buffer C is a compatible buffer for both the RE in our case. The reaction mixture was incubated overnight at 37°C. RNAase treatment of the plasmid if not performed during plasmid isolation then it can be incorporated in an RE digestion reaction as well (RNAase 1mg/ml 0.5µl). The enzymes used for the digestion were inactivated by heat treatment at 65°C for 20min. An aliquot (1µl) from the sample was run in 0.8% agarose and checked for a proper digestion before going for gel purification of the sample.
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3.2.2.6. Gel purification:
For recovering the fhuA insert and the double digested vector pUCPM18 vector 1.0% agarose was used and the gel purification method followed was as per Clean Genei Kit obtained from Bangalore Genei Pvt ltd, India.

3.2.2.7. DNA Ligation:
The purified insert was ligated into pUCPM18 vector. The ligation was set up as per the system described.

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity (µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Purified vector pUCPM18</td>
<td>3.0 µl</td>
</tr>
<tr>
<td>(~33ng/µl)</td>
<td></td>
</tr>
<tr>
<td>Purified insert fhuA (~14ng/µl)</td>
<td>12.0 µl</td>
</tr>
<tr>
<td>Ligase 40U/µl (1: 10) diluted</td>
<td>1.0 µl</td>
</tr>
<tr>
<td>10X Ligase buffer</td>
<td>2.0 µl</td>
</tr>
<tr>
<td>Sterile distilled water</td>
<td>2.0 µl</td>
</tr>
<tr>
<td>Total</td>
<td>20.0 µl</td>
</tr>
</tbody>
</table>

The ligation mixture was incubated overnight at 16°C since it was a cohesive end ligation. The amount of insert to be used for ligation was decided as per the formula:

\[
\text{Amount of insert (ng)} = \frac{\text{Amt of vector DNA} \times \text{Size of amplicon}}{\text{Size of vector}} \times (\frac{\text{Insert: Vector}}{\text{Molar ratio of}})
\]

The insert: vector ratio used is 4:1, Amount of plasmid DNA: 100ng, Size of the insert = 2.3kb, Size of the vector pUCPM18 = 5.3kb. Considering the above formula the amount of insert required is 160ng and the amount used for ligation was 168ng.
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CONTROLS

All the necessary controls along with the main ligation were kept under similar conditions.

- Double digested vector control (it should not get ligated as it is digested with BamHI and HindIII). Shows, that the double digestion, has taken place properly.
- Insert control (it should also not get ligated). Shows that insert has got digested properly.
- Mock ligation of single digested vector (it should get ligated). Shows that ligase is working.

3.2.2.8. Transformation of the ligated product:

The ligated products along with all the controls were transformed into E. coli DH5α. The competent cells were prepared using CaCl₂ method and heat shock method was used for transformation as mentioned by Sambrook and Russell, 2001. Before transforming the main ligated product, the purity of the competent cells was checked. A mock transformation was performed with known concentration of pBlueScript (pBS) and the efficiency of transformation was calculated. The ligated product was transformed only if the efficiency was ~ 10⁵ transformants/ml/µg of DNA.

3.2.2.9. Blue-White selection of the transformants:

The transformants were plated on Luria-Bertani (LB) agar plates containing ampicillin (100µg/ml) (antibiotic resistant marker present on pUCPM18) and 40µl of X-gal (20mg/ml). The plates were incubated at 37°C and results were observed after 18h. The blue colonies obtained were improperly digested vector molecules (those that were single digested with either of the enzymes). The white colonies (putative clones) were grid on a LB agar + Amp for future use.

3.2.2.10. Confirmation of the clones:

Plasmid DNA was extracted from overnight grown cultures of all the white colonies (putative clones) using the alkaline lysis miniprep protocol mentioned above. Plasmids were checked for the presence of the insert by RE digestion with EcoRI/HindIII and XbaI.
The digested product was analysed on a 1.0% agarose gel. Colonies showing the correct sizes of bands were preserved on LB agar + Amp for further experiments.

3.2.3 Phenotypic complementation of fhuA mutant MB97:

*E. coli* MB97, an *fhuA* deletion mutant was obtained as a kind gift from Prof. Volkmar Braun, Institute of Microbiology, University of Tuebingen, Germany. It was used for the phenotypic complementation study. In all the experiments, both the *E. coli* strains were induced with 0.4mM IPTG. Phenotypic complementation assay was performed by the following ways:

3.2.3.1 IPTG Induction:
The cultures were grown till O.D$_{600}$ 0.6; 0.4mM to 1mM IPTG was added to the cultures and was incubated at 37°C for 2-3h. These IPTG induced cultures were used for the further assays.

3.2.3.2 Ferrichrome bioassay:
Both *E. coli* MB97 and MB97::pGR1 were checked for cross-utilization of pure ferrichrome (50µg/ml, obtained from Sigma Aldrich Co.) in their respective minimum inhibitory EDTA plates. Procedure of bioassay was same as mentioned in 3.2.5.3. The plates were incubated at 37°C for 18-24h and results observed.

3.2.3.3 Sensitivity tests:
Antibiotic albomycin, microcin J25 and bacteriocin colicin M are all transported through *FhuA*; thus MB97::pGR1 was checked for its sensitivity to albomycin; microcin J25 and colicin M. The sensitivity assay was performed in a similar way to antibiotic sensitivity test. Both the cultures MB97 and MB97::pGR1 were spread on an LB plate and 8mm wells were bored using a sterile cork borer. Albomycin (50µg/ml), 100µl of microcin J25 (filter sterilized supernatant of *E. coli* strains containing pTUC203 mcjABCD) and 100µl of colicin M (filter sterilized supernatant of *E. coli* strains containing pTO4 cmacmi) were added into the wells. Albomycin and the *E. coli* strains containing pTO4 cmacmi and pTUC203
**Chapter 3 - Cloning and expression of *E. coli* fhuA gene in *C. cajan* rhizobial isolates:**

Effect of ferrichrome utilizing ability on the growth, nodulation and rhizospheric survivability under laboratory conditions.

*mcjABCD* were kindly gifted by Prof. Volkmar Braun, Institute of Microbiology, and University of Tuebingen, Germany. 100 μl of N-saline was added in the fourth well as a negative control.

3.2.3.4 Measurement of activity of albomycin and transport assay:

In order to determine the MIC of albomycin for MB97::pGR1, a range of albomycin concentrations between 0.1-1 μM (higher range) and 1-10 μM (lower range) was added in 8mm wells bored in LBA plates. The plates were seeded with 10^9 cells of *E. coli* MB97::pGR1. The inhibition zone was measured after incubation at 37°C overnight.

For transport assay the albomycin concentration used was slightly above the MIC. Here both the resistant (MB97: *fhuA* mutant cannot transport albomycin, so resistant) and sensitive (MB97::pGR1 containing *fhuA* therefore can transport albomycin within the cell and so sensitive) strain of *E. coli* were grown to 0.6 O.D₆₀₀. To detect albomycin transport an albomycin concentration of 1 μM was used; this exceeded the minimal inhibitory concentration (0.5 μM). To one set of 0.6 O.D₆₀₀ culture of sensitive strain 1 μM of albomycin was added and was incubated for various lengths of time (5 min, 10 min, 15 min and 25 min). Samples were withdrawn at intervals, chilled, centrifuged at 4°C and the remaining antibiotic activity in the medium/supernatant was determined by measuring the degree of inhibition of growth of *E. coli* MB97::pGR1 cells when incubated with this medium. Experiment with similar condition was performed with the resistant strain MB97 and was incubated with albomycin for 25 min. The strain MB97 and MB97::pGR1 grown without albomycin was used as controls.
3.2.3.5 Comparative OMP study:
The OMP profile was compared between the IPTG induced mutant and the transformant using the same protocol mentioned in section 3.2.8.1 and the extracts were run in 10% SDS-PAGE. The protein bands

3.2.4 Construction of transgenic rhizobial strains:
The construct pGR1 was transformed into the selected rhizobial strains by electroporation or conjugation. Rhizobial bioinoculant strain IC3123 was ampicillin sensitive and thus the pGR1 carrying ampicillin resistant marker was transformed into IC3123 by electroporation. Since electroporation did not work successfully in ST1 conjugation was used to transform it.

3.2.4.1 Sensitivity test of IC3123 and ST1:
The sensitivity of rhizobial strains IC3123 and ST1 against antibiotic albomycin, microcin J25 and bacteriocin colicin M was performed as mentioned in section 4.2.3.3.

3.2.4.2 Transformation of rhizobial bio-inoculant IC3123 and lab isolate ST1:
First the electro-competent cells were prepared. [Everything in electroporation was pre-chilled and ice-cold including the reagents and the electro-cuvette used for the procedure. All steps were performed at 4°C].

3.2.4.3 Competent cell preparation
For this 2% of overnight grown rhizobial culture was inoculated into 100ml of SOC medium and incubated at 28 ± 2°C. When its O.D600 reached around 0.9 the cells were harvested and were given several (five to six times) washes in 10% glycerol (ice-cold). At last the cells were resuspended in 0.5ml of 10% glycerol (ice-cold).

SOC medium: 10mM Magnesium Sulfate, 2.5mM KCl, 20mM Glucose, 10mM MgCl2. All the above composition was prepared in LB Broth.
3.2.4.4 Electroporation:

100µl electro-competent cells of ampicillin sensitive IC3123 was electroporated with 1µg of pGR1 carrying ampicillin resistant marker, at 12.5kV/cm (Electroporator 2510, Eppendorf). All the controls were properly taken care of.

<table>
<thead>
<tr>
<th>Culture</th>
<th>DNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>No DNA control</td>
<td>50 µl</td>
</tr>
<tr>
<td>Mock transformation</td>
<td>50 µl</td>
</tr>
<tr>
<td>Test transformation</td>
<td>50 µl</td>
</tr>
</tbody>
</table>

After electroporation, 1 ml of SOC medium was immediately added to the cultures. The cultures were then incubated at 37°C (30min at static condition and then 30min at shaking). After 1 hr the cultures were pelleted down and resuspended in 0.5ml SOC and 50µl was spread on LB plates containing ampicillin (100 mg/ml). The plates were incubated at 37°C for 24-48h. Rest of the sample was preserved at 4°C for further use.

3.2.4.5 Patch conjugation of ST1:

First kanamycin (40µg/ml) resistance was developed in ST1 parent that was sensitive to ampicillin (100µg/ml). The construct pGR1 was transformed into E. coli S17.1. ST1 Amp'Kan' was conjugated with S17.1:: pGR1 Amp'Kan' strain (Simon, 1984). Both the strains, that is the donor (S17.1:: pGR1 Amp'Kan') and the recipient (ST1 Amp'Kan') were grown separately till they reached an O.D<sub>600</sub> of 0.2. The donor and recipient were mixed in a ratio of 1:5 and pelleted down. The pellet was then made devoid of any antibiotic by washing it with saline. The mixed cultures were centrifuged and the pellet was dissolved in 50 µl sterile saline so as to form thick slurry. The slurry was placed onto a nitrocellulose membrane filter kept on a LB agar plate pre-warmed at 37°C. The system was incubated overnight at 37°C. The culture from the membrane was then resuspended in 10ml sterile saline, appropriate dilutions were made and the transconjugants were screened on an LB+Amp+Kan plates. Plating was done on LB agar plates containing 40µg/ml kanamycin, 100µg/ml ampicillin.
3.2.5 Confirmation of transgenic rhizobial strains:
The presence of the construct pGR1, in the rhizobial transformants was checked by isolation
of plasmid (pGR1) and its RE digestion with XbaI/HindIII. The isolated plasmid sample
and the RE digested samples were run on 1.0% agarose gel for confirming their presence.

3.2.6 Phenotypic expression of fhuA in the transgenic rhizobial strains
The rhizobial transformants ST1::pGR1 and IC3123::pGR1 were checked for the expression
of the fhuA gene first by ferrichrome utilization bioassay and for their sensitivity to
antibiotic albomycin; experiments were performed as mentioned in section 3.2.3.2.

3.2.6.1 Growth assay in presence of pure ferrichrome:
The growth of the parental and rhizobial transformant strains was compared in presence and
absence of pure ferrichrome. The cultures were grown in a medium containing siderophore
(ferrichrome added to a final concentration of 15μM). 1% inoculum of 1.0 O.D600 culture
was added to the growth system. All the necessary controls were maintained. The cultures
were allowed to grow under shaking conditions at 28±2°C. Samples were withdrawn every 4
hours and O.D600 was measured.

3.2.6.2 Effect of co-inoculation with U. maydis on the growth of rhizobial transformants:
The growth profiles of the transgenic rhizobial strains, the corresponding parent rhizobia and
U. maydis were compared (individually for all three) under deferrated conditions in nutrient
broth, by adding 0.5mM EDTA and their siderophore production was monitored using CAS
solution (Schwyn and Neilands, 1987). Growth profile of the transgenic rhizobial cultures
and the corresponding parent rhizobia was checked in presence and absence of Ustilago
maydis (ferrichrome producer) under iron starved conditions, using 0.5mM EDTA
concentration.

Construction of a transgenic Rhizobium for Cajanus cajan (pigeon pea)
By cloning and expression of a genomic region encoding iron siderophore receptor
For this, the parent *Rhizobium* sp. IC3123 and ST1 as well as the rhizobial transformant IC3123::pGRl and ST1::pGRl were grown individually. The log phase culture was inoculated into a fresh Nutrient Broth (NB) medium such that the initial count of each culture was $10^2$ cfu/ml. The four rhizobial strains mentioned above were co-inoculated separately with *Ustilago maydis* in NB such that the initial count of both the cultures individually was $10^2$ cfu/ml. These systems were incubated at $28\pm 2^\circ C$ under shaking condition. Aliquots were collected at a regular interval of 6h for 72 hrs. The samples were serially diluted and appropriate dilutions were plated on NB agar plates. The plates were incubated at $28\pm 2^\circ C$ for 48h to 72h and the cfu/ml was then calculated and the graph was plotted.

### 3.2.6.3 Comparative outer membrane protein study:

The parent and transgenic rhizobial cultures were grown in 250ml Ashby’s Glucose Broth (AGB) for 48h, under shaking conditions at 28 ± 2°C. CAS solution was used to check siderophore production. The culture was centrifuged at 8000 rpm for 30min to pellet down the cells which were resuspended in 5ml of 50mM Tris HCl pH 7.4 containing 0.5mM MgCl$_2$ and their OMP was extracted as mentioned in section 3.2.8.1. The OMP pellet was then dried and dissolved in sample buffer by mild heating and stored at -20°C until the samples were analyzed visually by running them on a 10% SDS-PAGE gel stained with coomassie blue R-250. Outer membrane protein profile was compared between the transgenic and their respective parent to confirm the expression.

### 3.2.7 In planta studies of the transgenic rhizobial strains:

Pigeon pea [BDN-2, Bharuch, Gujarat, India] seeds were surface sterilized by incubating in 0.1% HgCl$_2$. Surface sterilized seeds were allowed to germinate. Germinated seedlings were incubated in a thick suspension of overnight grown IC3123, IC3123::pUCPM18 (vector control), IC3123::pGR1, ST1, ST1::pUCPM18 (vector control) and ST1::pGRl cultures. Culture coated seedlings were sown in the in soil contained in pots. All the pot experiments were performed in triplicates. The systems were incubated in natural day-night conditions for 45 days. Watering was done with sterile water 2-3 times a day. After plant growth,
nodule number, root length, shoot length, root fresh weight, shoot fresh weight and chlorophyll content were calculated.

The nodules were checked for their nitrogenase activity by Acetylene Reduction Assay (ARA) and also for the presence of rhizobial transformants by crushing and plating on an antibiotic marker plate.

3.2.7.1 Chlorophyll Estimation: (Graan and Ort, 1984)

1g leaf was weighed and was washed thoroughly. It was kept in 4°C for 10-15min so that the leaf becomes turgid. The leaf was crushed in a mortar pistol in 80% acetone. The extract was filtered using a whatman filter no: 1. The filter paper was washed using 80% acetone. The volume was made equal to 100ml using volumetric flask. The absorbance was checked at 664nm and 647nm. Calculation of chlorophyll a, chlorophyll b and total chlorophyll in micro molar concentration was done using formula.

Chlorophyll was measured spectrophotometrically in 80% acetone using the specific absorption coefficients for chlorophyll a at 664nm and chlorophyll b at 647nm. The following equations were derived to give the micromolar concentration of chlorophyll a ($C_a$), chlorophyll b ($C_b$) and total chlorophyll.

$$C_a = 13.19 \times A_{664} - 2.57 \times A_{647}$$

$$C_b = 22.10 \times A_{647} - 5.26 \times A_{664}$$

$$\text{Total } (C_a + C_b) = 7.93 \times A_{664} + 19.53 \times A_{647}$$

3.2.7.2 Acetylene Reduction Assay (ARA):

Acetylene is reduced to ethylene by nitrogenase. The ethylene generated or produced was detected using gas chromatography (GLC) with Flame Ionization Detector (FID).

Plants were removed from the soil without disturbing the root nodules. The roots were excised with the nodules and the root system was placed into a 100ml conical flask. The flask was sealed with a rubber septum (serum cap). 10ml air was removed from the flask.
with an air tight syringe. 10ml acetylene was injected into the flask and incubated at room temperature (RT) for 2-3h. One to two ml of gas mixture was removed from the flask with an air tight syringe, injected into pre-conditioned GLC and ethylene and acetylene peaks monitored.

**GLC operating conditions:**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carrier Gas- Nitrogen with flow rate</td>
<td>30-45 ml/min</td>
</tr>
<tr>
<td>Gas for Detector- Hydrogen and Air</td>
<td>Porapak N, R, T and Q</td>
</tr>
<tr>
<td>Oven/ column temperature</td>
<td>60°C</td>
</tr>
<tr>
<td>Injection temperature</td>
<td>65°C</td>
</tr>
<tr>
<td>Detector temperature</td>
<td>85°C</td>
</tr>
<tr>
<td>Retention time for ethylene (min)</td>
<td>1.3</td>
</tr>
<tr>
<td>Retention time for acetylene (min)</td>
<td>1.8</td>
</tr>
</tbody>
</table>

**Generation of Acetylene:**

Calcium carbide (CaC₂) pellets (2-3) were added into 50ml of distilled water. After few seconds the system was capped with serum cap. Acetylene gas was sampled with an air tight syringe.

**3.2.7.3 Nodule Occupancy:**

The organisms within the nodules were screened on plates containing antibiotic marker for determining the nodule occupancy. Nodules were carefully removed from the roots, separately added into a sterile microfuge tube, 100µl YEM was added to each tube and nodules were aseptically crushed using pointed forceps and the extract was incubated for 12 h. After incubation, the root extract was streaked on AMB plates supplemented with appropriate concentration of antibiotics. The plates were incubated at 28±2°C and screened for growth in comparison to the control plates devoid of the antibiotics. The percentage nodule occupancy by the strain of interest was calculated as follows:
Chapter 3- Cloning and expression of E. coli fhuA gene in C. cajan rhizobial isolates: Effect of ferrichrome utilizing ability on the growth, nodulation and rhizospheric survivability under laboratory conditions.

\[ NO = 100 \frac{A}{B} \]

Where NO is the nodule occupancy in percent (%), A = No. of nodules carrying strain introduced by us, B = Total number of nodules tested.

3.3 RESULTS

3.3.1. Characterization of rhizobial bioinoculant strains

3.3.1.1. Siderophore production and cross utilization by the rhizobial bioinoculant strains and ST1:

Four rhizobial bioinoculant strains for pigeon pea were used for these studies. All the rhizobial bioinoculant strains as well as the laboratory isolate ST1 produced only catecholate type of siderophores (Table 3.2). Bioinoculant strains produced relatively less amount of siderophore ranging from 1-2 μg/ml while ST1 produced approximately ten times higher siderophore than the bioinoculant strains. Also the siderophore production ability of most of the natural isolates mentioned in previous chapter was better than the bioinoculant strains.

The minimum inhibitory EDTA concentration was found out for these strains and then they were tested for siderophore cross-utilization ability for some standard siderophores like 2-3, DHBA, ferric-citrate, desferal (100 μg/ml each) and ferrichrome, at a concentration of 50 μg/ml. The isolates showed a positive utilization test for 2-3, DHBA and ferric-citrate but were unable to utilize either desferal or ferrichrome (Table 3.2). These cultures were also checked for their ability to utilize siderophores produced by *Pseudomonas aeruginosa*, *P. fluorescens*, *P. putida* and *Rhodotorula mucilaginosa* all of which produce different types of hydroxamate siderophores. *P. aeruginosa*, *P. fluorescens* and *P. putida* are known to produce pyoverdine like siderophores and *R. mucilaginosa* is known to produce rhodotorulic acid (Andersen, et al., 2003). None of the rhizobial isolates could utilize the siderophores produced by *P. aeruginosa* and *R. mucilaginosa*; however the ability to utilize siderophores of *P. fluorescens* and *P. putida* varied. IC3109 and IC3163 utilized siderophore produced by *P. fluorescens* and the rhizobial isolates IC3123, ST1 and IC3169 utilized siderophore produced by *P. putida* (Table 3.2).
Table 3.2: Siderophore production and utilization by C. cajan rhizobial bioinoculant strains.

<table>
<thead>
<tr>
<th>Rhizobial strains (MIC EDTA)</th>
<th>Siderophore production [μg/ml] (type of siderophore)</th>
<th>Siderophore Utilization</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Fe-cit 2-3, DHBA Desf FC PA PF PP RM</td>
<td></td>
</tr>
<tr>
<td>IC3109 (3.0mM)</td>
<td>1.342 (C)</td>
<td>+ + - - - + -</td>
</tr>
<tr>
<td>IC3123 (3.5mM)</td>
<td>2.376 (C)</td>
<td>+ + - - - + -</td>
</tr>
<tr>
<td>IC3163 (3.0mM)</td>
<td>2.376 (C)</td>
<td>+ + - - - + -</td>
</tr>
<tr>
<td>IC3169 (3.5mM)</td>
<td>2.046 (C)</td>
<td>+ + - - - + -</td>
</tr>
<tr>
<td>ST1 (5.0mM)</td>
<td>16.5 (C)</td>
<td>+ + - - - + -</td>
</tr>
</tbody>
</table>

C: Catecholate type siderophore; Fe-cit: Ferric citrate; 2-3, DHBA: 2-3, Dihydroxybenzoic acid; Desf: Desferrioxamine B; FC: Ferrichrome; PA: P. aeruginosa culture supernatant; PF: P. fluorescens culture supernatant; PP: P. putida culture supernatant; RM: Rhodotorula mucilaginosa 850 culture supernatant; + indicates growth and - no growth around the siderophore containing wells. MIC: Minimum Inhibitory Concentration

Additionally it was observed that IC3109 and IC3163 at 1.5 to 2.0mM EDTA showed a zone of inhibition (ZOI) against siderophore of P. aeruginosa and P. putida respectively (data not shown). Under these conditions both the test cultures showed a confluent growth on the plates except around the wells containing siderophore. This suggested that the siderophores of IC3109 and IC3163 were probably weaker than the siderophores of Pseudomonas spp. and thus could not quench the Fe³⁺ from them and this resulted in ZOI. Based on these observations, rhizobial strains IC3123, IC3169 and ST1 were taken forward for further studies for cloning of ferrichrome receptor.
3.3.2 16SrRNA gene amplification and sequencing of selected isolates:
To confirm the identity of the rhizobial bioinoculant strains selected for further studies the 16SrRNA of all the three strains was amplified using the universal eubacterial primers and a partial sequence of the amplicon was obtained and sequenced in Bangalore Genei. Pvt ltd, India (Fig 3.1). Their 16S rRNA partial gene sequence of the isolates IC3123 (Genbank Accession number DQ632607) and ST1 (Genbank Accession number DQ632608) showed to bear 95 % (Fig 3.2) and 96.7 % (Fig 3.3) sequence similarity with *Rhizobium spp* respectively. IC3169 (Genbank Accession number EF211976) showed 98.6% (Fig 3.4) sequence similarity with *Sinorhizobium spp*. Since *Sinorhizobium meliloti* is known to have *fhuA* homologues therefore this strain (IC3169) was not taken ahead for the cloning and expression of *fhuA*. Both the rhizobial strains (IC3123 and ST1) were also tested for their ability to nodulate *C. cajan*. Both were able to form fresh and healthy nodules within 55-60 days and 40-45 days respectively (Fig 3.5).
Chapter 3 - Cloning and expression of E. coli 1Beta gene in C. cajan rhizobial isolates: Effect of ferrichrome utilizing ability on the growth, nodulation and rhizospheric survivability under laboratory conditions.

A. Sequence data of IC3123

GCACATCTCACGAACACGCTGACGACAGCCATGACCTAAGCTGACAGGAGCAGCAGGAGCTGACGACTGACAGCCATGCAGCACCTGTTCTGGGGCCAGCCTAACTGAAGGACATCGTCTCCAATGCCCATACCCCGAATGTCAAGAGCTGGTAAGGTTCTGCGCGTTGCTTCGAATTAAACCACATGCTCCACCGCTTGTGCGGGCCCCCGTCAATTCCTTTGAGTTTTAATCTTGCGACCGTACTCCCCAGGCGGAATGTTTAATGCGTTAGCTGCGCCACCGAACAGTATACTGCCCGACGGCTAACATTCATCGTTTACGGCGTGGACTACCAGGGTATCTAATCCTGTTTGCTCCCCACGCTTTCGCACCTCAGCGTCAGTAATGGACCAGTAAGCCGCCTTCGCCACTGGTGTTCCTCCGAATATCTACGAATTTCACCCTTACACTCGGAATTCCACTTACCTCTTCCATACTCAAGATACCCAGTATCAAAGGCAGTTCCGCAGTTGAGCTGCGGGATTTCACCCCTGACTTAAATATCCGCCTACGTGCGCTTTACGCCCAGTAATTCCGAACAACGCTAGCCCCCTTCGTATTACCGCGGCTGCTGGCACGAAGTTAGCCGGGGCTTCTTCTCCGACCTACCGTCATTATCTTCATCGGTGAAAGAGCTTTACAGCCCTAAGCCTTCATCACTCACGCGGCATGGCTGGATCAGGCTTGCCGCAATTGTCCAATATTCCCCACTGCTGCCTCCGTAGGAGTTTGGGCCGTGTCTCAGTCCCATGTGGCAGTGCATCCTCTCAACAGCTATGGATCGTCGCTTGTAGCTTTACCCCACCACTAGCTATCACGGGGCCATCCTTCGGAATCTTCCCCGTAGGGGAGGGGATAATC

B. Sequence data of ST1

CTCGAATGGCACACTGGTGGGTCCTGTTCTGGGGCAGCCTAACTGAAGGACATCGTCTCCAATGCCCATACCCCGAATGTCAAGAGCTGGTAAGGTTCTGCGCGTTGCTTCGAATTAAACCACATGCTCCACCGCTTGTGCGGGCCCCCGTCAATTCCTTTGAGTTTTAATCTTGCGACCGTACTCCCCAGGCGGAATGTTTAATGCGTTAGCTGCGCCACCGAACAGTATACTGCCCGACGGCTAACATTCATCGTTTACGGCGTGGACTACCAGGGTATCTAATCCTGTTTGCTCCCCACGCTTTCGCACCTCAGCGTCAGTAATGGACCAGTAAGCCGCCTTCGCCACTGGTGTTCCTCCGAATATCTACGAATTTCACCCTTACACTCGGAATTCCACTTACCTCTTCCATACTCAAGATACCCAGTATCAAAGGCAGTTCCGCAGTTGAGCTGCGGGATTTCACCCCTGACTTAAATATCCGCCTACGTGCGCTTTACGCCCAGTAATTCCGAACAACGCTAGCCCCCTTCGTATTACCGCGGCTGCTGGCACGAAGTTAGCCGGGGCTTCTTCTCCGACCTACCGTCATTATCTTCATCGGTGAAAGAGCTTTACAGCCCTAAGCCTTCATCACTCACGCGGCATGGCTGGATCAGGCTTGCCGCAATTGTCCAATATTCCCCACTGCTGCCTCCGTAGGAGTTTGGGCCGTGTCTCAGTCCCATGTGGCAGTGCATCCTCTCAACAGCTATGGATCGTCGCTTGTAGCTTTACCCCACCACTAGCTATCACGGGGCCATCCTTCGGAATCTTCCCCGTAGGGGAGGGGATAATC

C. Sequence data of IC3169

CTTAACACATGCAAGTCGAGCGCGTAGCAATACGAGCGGCAGACGGGTGAGTAACGCGTGGGAATCTACCCTTTTCTACGGAATAACGCAGGGAAACTTGTGCTATACCGTATACGCCCTTTGGGGGAAAGATTATCGGAGAAGGATGAGCCCGCGTTGGATTAGCTAGTTGGTGGGGTAAAGGCCTACCAAGGCGACGATCCATAGCTGGTCTGAGAGGATGATCAGCCACATTGGGACTGAGACACGGCCCAAACTCCTACGGGACAGCAGCAGTGGGGAATATTGGACAATGGGCGCAAGCCTGATCCAGCCATGCCGCGTGAGTGATGAAGGCCCTAGGGTTGTAAAGCTCTTTC

Fig 3.1 The 16s rDNA sequence data of Rhizobium sp. IC3123 (A), ST1 (B) and Sinorhizobium sp. IC3169 (C).
Chapter 3- Cloning and expression of E. coli fluA gene in C. cajan rhizobial isolates: Effect of ferrichrome utilizing ability on the growth, nodulation and rhizospheric survivability under laboratory conditions.

**Fig 3.2 Gen Bank accession result of partial 16S rRNA gene sequence of rhizobial bioinoculant strain IC3123**
Chapter 3 - Cloning and expression of E. coli fhuA gene in C. cajan rhizobial isolates: Effect of ferrichrome utilizing ability on the growth, nodulation and rhizospheric survivability under laboratory conditions.

Fig 3.3 Gen Bank accession result of partial 16S rRNA gene sequence of rhizobial lab isolate ST1
Chapter 3: Cloning and expression of E. coli fhuA gene in C. cajan rhizobial isolates:
Effect of ferrichrome utilizing ability on the growth, modulation and rhizospheric survivability under laboratory conditions.

Fig 3.4 Gen Bank accession result of partial 16S rRNA gene sequence of rhizobial bioinoculant strain IC3169

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3.3.3 Antibiotic sensitivity test of the selected isolates

In order to carry out transformation or conjugation with rhizobial isolates it is important to understand the antibiotic resistance pattern of the rhizobial isolates for effective screening of the transconjugants/ transformants with suitable antibiotics. The rhizobial isolates were tested for their sensitivity towards the antibiotics in the Octadisc (OD 043) and separate antibiotic discs were used for Neomycin and Rifampicin. The rhizobial test isolates IC3123 and ST1 were sensitive to all the antibiotics on the octadisc and to Neomycin and Rifampicin. The sensitivities of the test rhizobia for the antibiotics that were used are shown in Table 3.3. Kanamycin resistance upto 40µg/ml was developed in the above strains to further use them for conjugation.
Table 3.3 Antibiotic sensitivity of *Rhizobium* sp. ST1 and IC3123

<table>
<thead>
<tr>
<th>Antibiotics (symbol) conc.</th>
<th>IC3123</th>
<th>ST1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chloramphenicol (C) 30</td>
<td>S</td>
<td>S</td>
</tr>
<tr>
<td>Ampicillin (A)-10</td>
<td>S</td>
<td>S</td>
</tr>
<tr>
<td>Tetracycline (T)-30</td>
<td>S</td>
<td>S</td>
</tr>
<tr>
<td>Gentamycin (G)-10</td>
<td>S</td>
<td>S</td>
</tr>
<tr>
<td>Kanamycin (K)-30</td>
<td>S</td>
<td>S</td>
</tr>
<tr>
<td>Co-trimozole (Co)-25</td>
<td>S</td>
<td>R</td>
</tr>
<tr>
<td>Amikacin (Ak)-30</td>
<td>S</td>
<td>S</td>
</tr>
<tr>
<td>Streptomycin (S)-25</td>
<td>S</td>
<td>S</td>
</tr>
<tr>
<td>Neomycin (N)-10</td>
<td>S</td>
<td>S</td>
</tr>
<tr>
<td>Rifampicin (R)-05</td>
<td>S</td>
<td>S</td>
</tr>
</tbody>
</table>

S: indicates sensitivity  
R: indicates resistance

3.3.4 Construction of the *fhuA* expression vector pGR1:

Sub-cloning *fhuA* gene of *E. coli* in pUCPM18 expression vector

Plasmid pET17b containing *E. coli* *fhuA* gene of 2.3kb (gifted by Dr. Ranjan Chakraborty, East Tennessee State University, USA) was double digested using the restriction enzymes *BglII/HindIII* and ligated to 5.3kb pUCPM18 (Hester et al, 2000) double digested with *BamHI/HindIII* (Fig 3.6) in which the expression of *fhuA* was driven under the *lac* promoter of *E. coli*.
Fig 3.6: Schematic representation of the sub-cloning strategy used to clone fluA in pUCPM18-Gm
3.3.5 Confirmation of the E. coli clones obtained

The ligated product was transformed into E. coli DH5α and the white colonies obtained on LB-Xgal plates were checked for the presence of pGR1 by isolating and digesting the plasmid obtained with EcoRI/HindIII. Two out of first eight clones tested were successful in giving the expected band size of 2.3kb and 5.3kb (Fig 3.7a). Many such clones were screened and we obtained about 30 positive clones in 109 tested, showing 30% efficient ligation. These clones were further reconfirmed by digesting their plasmid with XbaI/HindIII, XbaI site being present only in the 2.3kb fhuA insert. All the positive clones were successful in giving the expected band size (2.3kb +5.3kb) when digested with XbaI/HindIII (Fig 3.7b shows the pattern for a representative clone).

Fig 3.6a: RE digestion of the plasmid obtained from putative clones (pGR1) with EcoRI/HindIII. Clone 1(lane 1), clone 2 (lane 2), clone 3 (lane 3), clone 4 (lane 4), clone 5 (lane 5), clone 6 (lane 6), clone 7 (lane 7), clone 8 (lane 8). Lane 9 shows the marker of λ DNA Hind III digest. Clones 1 and 4 were positive in showing the expected bands of 5.3 and 2.3kb.

Fig 3.6b: RE digestion of the ligated product pGR1 with XbaI/HindIII (lane 1). Controls of only vector pUCPM18 digested with XbaI/HindIII (lane 2), eluted insert 2.3kb (lane 3), and eluted vector 5.3 kb (lane 5) were also added. Lane 4 shows the marker of λ DNA Hind III digest.
3.3.6 Phenotypic complementation of \textit{fhuA} delta mutant MB97

It was important to check whether the \textit{fhuA} gene of pGR1 construct is functional. This was confirmed in \textit{E. coli} by transformation of pGR1 in strain MB97 (\textit{fhuA} deletion mutant) and checking its phenotypic complementation. In all the experiments the results of parent MB97 strain was compared with the transformed strain MB97::pGR1.

3.3.6.1 Comparative OMP study:

In order to detect the presence of the \textit{fhuA} protein in the outer membrane of MB97::pGR1 the outer membrane protein preparation of both MB97 and MB97::pGR1 compared. It was seen that in the transformant MB97::pGR1 there was a clear induction of an approximately 78kDa protein upon IPTG addition that was absent in the parent strain (Fig 3.8) indicating the expression of the \textit{fhuA}.

![Fig. 3.8: Outer membrane protein profiles of \textit{E. coli} MB97::pGR1 (lane 2) and \textit{E. coli} MB97 (lane 3) when induced with 0.4mM IPTG. Arrow head indicates band of approximately 78 kDa specifically induced in plasmid containing strain.](image)

3.3.6.2 Ferrichrome Bioassay:

After confirming that the \textit{fhuA} gene was getting expressed it was essential to know whether it was functional in the \textit{E. coli} system. For this first of all, the MB97::pGR1 transformant was checked for the ferrichrome cross-utilization and it was observed that the \textit{fhuA}
transformants showed positive ferrichrome cross-utilization whereas the parent strain was unable to do so (Fig 3.9). This confirmed that the gene was functional.

![Fig 3.9: Ferrichrome bioassay (50μg/ml) of MB97ΔfhuA mutant (A) and its transformant MB97::pGR1 (B)](image)

3.3.6.3 Sensitivity test:
Since FhuA receptor is also known to transport antibiotics like albomycin and microcin J25 and one of the bacteriocin called colicin M, the successful expression of this gene in MB97 was hypothesized to cause growth inhibition upon application of these antimicrobial agents. Thus the functional expression was reconfirmed by checking the effect of these agents on MB97 and MB97::pGR1. A clear zone of inhibition of MB97::pGR1 was observed when albomycin and colicin M was added in the wells and weak inhibition was seen in case of microcin J25, whereas no such inhibition was observed in MB97 (Fig 3.10). This confirmed that the \textit{fhuA} gene was getting expressed in \textit{E. coli} MB97.

![Fig 3.10: Effect of addition of microcin J25, colicin M, albomycin on \textit{E. coli} (A) MB97ΔfhuA mutant and on (B) MB97::pGR1 transformant. N-Saline was taken as control.](image)
3.3.6.4 Transport of Albomycin through FhuA receptor of MB97::pGR1:
The basic idea of this experiment was that MB97::pGR1 containing the FhuA receptor when incubated with albomycin would efficiently transport it into the cell. Thus, as the time of incubation increased the amount of albomycin in the supernatant of this system should decrease and thus a sensitive strain (wild type E. coli) when inoculated in this supernatant (with the left over albomycin after transport) should show an increase in the growth and survival with increasing incubation time. Accordingly in this experiment it was seen that after 5 min and 10 min incubation of MB97::pGR1 with albomycin there was a gradual decrease in the inhibitory activity of albomycin present in the supernatant on the growth of the sensitive E. coli strain (Fig 3.11), showing that the albomycin had been removed from the medium due to its uptake by MB97::pGR1 with the increase in time of incubation. It could be seen that in 25 min incubation with albomycin there was a complete recovery of growth profile showing that probably there was no albomycin left in the medium after 25 min (Fig 3.11).

Albomycin remained fully active when incubated with fhuA mutant MB97 for 25 min showing indicating that the antibiotic added remained in the supernatant and hence caused growth inhibition of the sensitive E. coli strain.
Fig 3.11: Growth assay of sensitive *E. coli* strain when grown in the presence of supernatant obtained after the treatments given in the block in the right.

All the above experiments proved that the *fhuA* construct was functionally expressed in the self system. Thus we proceeded with the transformation of the selected rhizobial strains IC3123 and ST1 with pGR1.

### 3.3.7 Construction and confirmation of rhizobial transformants carrying *pGR1*:

The pGR1 plasmid was introduced into *Rhizobium sp.* IC3123 by electroporation (12.5kV/cm) and *Rhizobium sp.* ST1 by patch mating with *E. coli* S17.1 carrying pGR1. The rhizobial transformants were called IC3123::pGR1 and ST1::pGR1 respectively. Thus pGR1 was first transformed into S17.1 and then this S17.1:: pGR1 was used for further conjugation with ST1. The transformants and transconjugants obtained were grown under respective antibiotic stress and further confirmed by plasmid isolation and RE digestion with *XbaI* and *HindIII*. The selected transformants showed the presence of plasmid and when digested gave bands of required molecular weights of 2.3kb (*fhuA*) and 5.3kb (vector backbone) (Fig 3.12).
3.3.8 Phenotypic expression of pGR1 in Rhizobium species IC3123::pGR1 and ST1::pGR1

3.3.8.1 Comparative outer membrane protein study

The outer membrane proteins of IC3123::pGR1 and ST1::pGR1 were extracted and compared with the parent rhizobial strains to see the expression of \textit{fhuA} gene in rhizobia. Both the transformant strains showed the presence of an extra 78kDa band that was absent in the parent strain (Fig 3.13). This showed that the \textit{fhuA} gene was expressed and the receptor protein was successfully produced and transported to outer membrane.
Chapter 3- Cloning and expression of E. coli fhuA gene in C. cajan rhizobial isolates: Effect of ferrichrome utilizing ability on the growth, nodulation and rhizospheric survivability under laboratory conditions.

Fig 3.13: Outer membrane protein profiles of *Rhizobium* sp. under deferrated conditions (A) IC3123::pGRl (lane 1) and IC3123 (lane 2) with protein molecular marker (lane 3) (B) ST1 (lane 1) and ST1::pGRl (lane 3) with protein molecular marker (lane 1) performed in 10% SDS-PAGE. The arrow indicates a 78kDa protein band that is exclusively seen in the transformants.

3.3.8.2 Ferrichrome bioassay and sensitivity to antibiotic albomycin

As the rhizobial transformants were successful in expressing the FhuA receptor protein, it was essential to check whether the protein expressed was functional or not. For this we checked the ferri-ferrichrome utilization by IC3123::pGRl and ST1::pGRl. Additionally, transformants were also checked for their sensitivity towards antibiotic albomycin. Both the transformants showed a clear zone of exhibition when checked for ferrichrome utilization (Fig 3.14). Both the rhizobial strains IC3123::pGRl and ST1::pGRl were unable to show zone of inhibition with albomycin but instead of that they produced a turbid zone (Fig 3.15). This could be because the antibiotic probably imparted a bacteriostatic effect on these rhizobial strains rather than bactericidal effect.
Chapter 3- Cloning and expression of E. coli shuA gene in C. cajan rhizobial isolates : Effect of ferrichrome utilizing ability on the growth, nodulation and rhizospheric survivability under laboratory conditions.

Fig 3.14: Ferrichrome (50µg/ml) utilization by rhizobial transformants. Parent rhizobial strains ST1 and IC3123 do not show zone of exhibition whereas the transformants ST1::pGR1 and IC3123::pGR1 do.

Fig 3.15: Effect of albomycin on *Rhizobium* sp. (A) IC3123; (B) IC3123::pGR1; (C) ST1 and (D) ST1::pGR1. Transformants show sensitivity to the antibiotic.
All the above experiments showed that pGR1 carrying \(fhuA\) gene was getting expressed and was also functionally active in a non-self system like rhizobia. In the nature it is not only important that the rhizobial bioinoculants have the ability to utilize heterologous siderophores but it is also essential that this ability imparts them a growth and survival advantage.

3.3.8.3 Growth studies of rhizobial \(fhuA\) transformants in presence of ferrichrome:
In order to check whether ferrichrome utilizing ability provided the rhizobial transformants growth advantage or not, both the rhizobial strains IC3123::pGR1 and ST1::pGR1 were grown in presence and absence of pure ferrichrome (15\(\mu\)M) and compared with their parent strains. Both ST1::pGR1 and IC3123::pGR1 showed growth stimulation in presence of pure ferrichrome (Fig 3.16B, 3.17B), whereas in case of the parent \(Rhizobium\) sp. ST1 (Fig 3.16A) and IC3123 (Fig 3.17A), there was no significant difference in the growth profile in presence and absence of ferrichrome.

3.3.8.4 Growth studies of rhizobial \(fhuA\) transformants coinoculated with \(U.\ maydis\):
The growth stimulation observed in the rhizobial transformants in presence of ferrichrome was further confirmed by performing a co-inoculation experiment where the rhizobial transformants and respective parental strains were grown in presence of \(U.\ maydis\) (a ferrichrome producer). Both the rhizobial transformant ST1::pGR1 and IC3123::pGR1 showed increase in growth in terms of cfu/ml in presence of \(U.\ maydis\) (Fig 3.18B, 3.19B), whereas no such increase was observed in the growth of parent rhizobial strains ST1 and IC3123 (Fig 3.18A, 3.19A). These results were in coordination with the earlier results of FC cross-utilization, confirming the expression of FC receptor FhuA in IC3123::pGR1 and ST1::pGR1.

This experiment showed that even in the presence of culture which produced ferrichrome as a siderophore (a condition generally pertinent in soil), rhizobial \(fhuA\) transformants were able to increase in number as compared to the original parent strains. This suggested that the transgenic rhizobial bioinoculants may survive better under natural environments present in soil.
Construction of a transgenic Rhizobium for Cajanus cajan (pigeon pea) 
By cloning and expression of a genomic region encoding iron siderophore receptor

Chapter 3 - Cloning and expression of E. coli fhuA gene in C. cajen rhizobial isolates: 
Effect of ferrichrome utilizing ability on the growth, nodulation and 
rhizospheric survivability under laboratory conditions.

Fig 3.16: Growth of Rhizobium sp. ST1 (A) and ST1::pGR1 (B) in presence and absence of 
externally supplemented ferrichrome (15μM) indicated as +FC. Data are presented as the mean of 
three independent experiments and vertical bars indicate standard deviation.
Fig 3.17: Growth of *Rhizobium* sp. IC3123 (A) and IC3123::pGR1 (B) in presence and absence of externally supplemented ferrichrome (15μM) indicated as +FC. Data are presented as the mean of three independent experiments and vertical bars indicate standard deviation.
Chapter 3: Cloning and expression of \( E. \ coli \) fimA gene in \( C. \ cajan \) rhizobial isolates: Effect of ferrichrome utilizing ability on the growth, nodulation and rhizospheric survivability under laboratory conditions.

Fig 3.18: Growth of \( Rhizobium \) sp. ST1 (A) and ST1::pGR1 (B) in presence and absence of \( U. \ maydis \) (Um) under deferrated conditions. Data are presented as the mean of three independent experiments and vertical bars indicate standard deviation.
Chapter 3: Cloning and expression of \( E. coli \) flhA gene in \( C. cajan \) rhizobial isolates: Effect of ferrichrome utilizing ability on the growth, modulation and rhizospheric survivability under laboratory conditions.

**Fig 3.19:** Growth of bioinoculant *Rhizobium* sp. IC3123 (A) and IC3123::pGR1 (B) in presence and absence of *U. maydis* (Um) under deferrated conditions. Data are presented as the mean of three independent experiments and vertical bars indicate standard deviation.

Construction of a transgenic *Rhizobium* for *Cajanus cajan* (pigeon pea) by cloning and expression of a genomic region encoding iron siderophore receptor
3.3.9 Plant inoculation studies of pigeon pea with rhizobial fhuA transformants under autoclaved conditions:

3.3.9.1 Pot inoculation studies were performed in order to compare the abilities of the parent and the transformed strains to colonize plant roots in soil conditions and their subsequent effect on the plant growth. Pigeon pea plants were harvested and examined after approximately 7-8 weeks of inoculation with *Rhizobium sp.* ST1, ST1::pGR1, IC3123 and IC3123::pGR1 under autoclaved soil conditions. Both the transformants i.e., IC3123::pGR1 (42.82% increase or 1.42 fold) and ST1::pGR1 (50.09% increase or 1.5 fold) showed approximately 1.5 fold increase in the nodule number per plant as compared to the parental strains. In conditions where *U. maydis* was co-inoculated, IC3123::pGR1 (56.95% increase) and ST1::pGR1 (106.37% increase) showed 1.56 fold and 2 fold increase in nodule number per plant respectively (Fig 3.20E). A significant increase was also shown by the transformed strains in the root length (Fig 3.20B), shoot fresh weight (Fig 3.20C), root fresh weight (Fig 3.20D) and chlorophyll content (Fig 3.20F). Thus the engineered strains showed a clear benefit in terms of overall plant growth.

3.3.9.2 Rhizospheric colonization was checked after 15 days of plant growth and it was clearly seen that transformed strains were able to survive better in soil as compared to the parents. The starting concentration of IC3123 and IC3123::pGR1 in the soil was approximately $6.2 \times 10^3 \text{ cfu/g}$ and $7 \times 10^3 \text{ cfu/g}$ rhizospheric soil respectively. But after 15 days of postinoculation in case of the IC3123::pGR1 the concentration was $9 \times 10^6 \text{ cfu/g}$ of soil whereas in case of IC3123 it was only $5 \times 10^4 \text{ cfu/g}$ of soil. The initial number of both ST1 and ST1::pGR1 was approximately $5 \times 10^4 \text{ cfu/g}$ rhizospheric soil and after 15 days in case of the ST1::pGR1 the concentration was $2 \times 10^6 \text{ cfu/g}$ of soil whereas in case of ST1 it was only $3 \times 10^5 \text{ cfu/g}$ of soil.

3.3.9.3 Acetylene reduction assay (ARA) of all the nodules from each plant system showed positive nitrogenase activity as evidenced by the reduction of acetylene (retention time 1.8min) to ethylene (retention time 1.3min) depicted in the GC chromatogram (Fig 3.21).
Chapter 3 - Cloning and expression of E. coli flaA gene in C. cajan rhizobial isolates: Effect of ferrichrome utilizing ability on the growth, nodulation and rhizospheric survivability under laboratory conditions.

A

![Graph showing shoot length comparison](image)

B

![Graph showing root length comparison](image)

Construction of a transgenic Rhizobium for Cajanus cajan (pigeon pea) by cloning and expression of a genomic region encoding iron siderophore receptor.
Chapter 3: Cloning and expression of E. coli fisH1 gene in C. cajan rhizobial isolates: Effect of ferrichrome utilizing ability on the growth, nodulation and rhizospheric survivability under laboratory conditions.

C

D

Construction of a transgenic Rhizobium for Cajanus cajan (pigeon pea) By cloning and expression of a genomic region encoding iron siderophore receptor
Fig 3.20 A-F Effect of rhizobial transformants on the growth of pigeon pea plant in presence and absence of *U. maydis* (UM) under autoclaved soil conditions. Parent bioinoculant strains were used as control. All the treatments were performed in triplicates.
Chapter 3: Cloning and expression of E. coli fhuA gene in C. cajan rhizobial isolates: Effect of ferrichrome utilizing ability on the growth, nodulation and rhizospheric survivability under laboratory conditions.

**Fig 3.21:** The ARA activities of nodules formed by the rhizobial transformants as well as parents under autoclaved soil condition. Peak at 1.3min denotes ethylene and at 1.8min shows the presence of acetylene in GC.

Acetylene peak

Construction of a transgenic Rhizobium for Cajanus cajan (pigeon pea) by cloning and expression of a genomic region encoding iron siderophore receptor
3.3.10 Plant inoculation studies of pigeon pea with rhizobial fhuA transformants under unautoclaved conditions:

In plant studies performed under unsterilized soil conditions (Fig 3.22), the pigeon pea seedlings inoculated with fhuA transformants produced significant increase in shoot and root fresh weight, nodule density and chlorophyll content of the leaves as compared to parent strains or the uninoculated control plants. In certain plants there was some amount of growth enhancement when the transformant was co-inoculated with U. maydis but most of the plants showed similar profile even in presence and absence of U. maydis (Fig 3.23 A-F).

Occupancy of the nodules by IC3123::pGR1 and ST1::pGR1 was calculated by screening the culture within nodules, on antibiotic marker plates and it was calculated to be 73% and 75% respectively, whereas the nodules occupied by the parent rhizobial strains IC3123 and ST1 were 55% and 50% only. However, co-inoculation of U. maydis with IC3123::pGR1 and ST1::pGR1 increased the nodule occupancy upto 86% and 79% respectively (Table 3.4).
Chapter 3 - Cloning and expression of $E. coli$ fhuA gene in C. cajan rhizobial isolates: Effect of ferrichrome utilizing ability on the growth, nodulation and rhizospheric survivability under laboratory conditions.

Fig 3.22: Pot studies of plants treated with wild type *Rhizobium sp. IC3123*, transformant IC3123::pGR1 (A) and with wild type *Rhizobium sp. ST1*, transformant ST1::pGR1 (B) in presence and absence of *U. maydis* under unautoclaved soil conditions.

Table 3.4: Percentage of nodules occupied by the inoculated strains of rhizobia and their fhuA transformants under natural (unautoclaved) soil conditions

<table>
<thead>
<tr>
<th>Culture inoculated</th>
<th>Total no of nodules/ plant</th>
<th>% of nodules occupied by organism of interest</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>11</td>
<td>-</td>
</tr>
<tr>
<td>Um alone</td>
<td>13</td>
<td>-</td>
</tr>
<tr>
<td>IC3123</td>
<td>29</td>
<td>55.17</td>
</tr>
<tr>
<td>IC3123::pGR1</td>
<td>34</td>
<td>73.52</td>
</tr>
<tr>
<td>IC3123::pUCPM18</td>
<td>28</td>
<td>46.42</td>
</tr>
<tr>
<td>IC3123+Um</td>
<td>21</td>
<td>57.14</td>
</tr>
<tr>
<td>IC3123::pGR1+Um</td>
<td>44</td>
<td>86.36</td>
</tr>
<tr>
<td>ST1</td>
<td>38</td>
<td>50.00</td>
</tr>
<tr>
<td>ST1::pGR1</td>
<td>44</td>
<td>75.00</td>
</tr>
<tr>
<td>ST1::pUCPM18</td>
<td>36</td>
<td>61.11</td>
</tr>
<tr>
<td>ST1+ Um</td>
<td>38</td>
<td>34.21</td>
</tr>
<tr>
<td>ST1::pGR1+ Um</td>
<td>53</td>
<td>79.24</td>
</tr>
</tbody>
</table>
Chapter 3: Cloning and expression of E. coli fimH gene in C. cajan rhizobial isolates: Effect of ferrichrome utilizing ability on the growth, nodulation and rhizospheric survivability under laboratory conditions.
Chapter 3: Cloning and expression of E. coli hau gene in C. cajan rhizobial isolates: Effect of ferrichrome utilizing ability on the growth, nodulisation and rhizospheric survivability under laboratory conditions.

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Construction of a transgenic Rhizobium for Cajanus cajan (pigeon pea) by cloning and expression of a genomic region encoding iron siderophore receptor

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**Fig 3.23:** A-F Effect of rhizobial transformants on the growth of pigeon pea plant in presence and absence of *U. maydis* under unautoclaved soil conditions. Parent bioinoculant strains were used as control. All the treatments were performed in triplicates.

**Chapter 3- Cloning and expression of *E. coli fhuA* gene in *C. cajan* rhizobial isolates: Effect of ferrichrome utilizing ability on the growth, nodulation and rhizospheric survivability under laboratory conditions.**

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*Construction of a transgenic *Rhizobium* for *Cajanus cajan* (pigeon pea) by cloning and expression of a genomic region encoding iron siderophore receptor*  
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3.4 DISCUSSION

The rhizosphere as an ecosystem consists of microbial populations engaged in competitive interactions for limiting nutrients, iron being one of them (Somers and Srinivasan, 2004). Interest in the iron uptake mechanisms of root nodule bacteria emerges from the understanding that iron-containing proteins are prominent members of the nitrogen-fixing machinery (Guerinot 1991). Rhizobial bioinoculant strains used in the present investigation were found to produce and utilize catecholate type siderophores but were poor producers as well as utilizers of hydroxamate-type of siderophores. Similar findings were made with native *C. cajan* root nodule isolates obtained from local fields (Khan et al., 2006). However, hydroxamate siderophores are known to be present in soil at high concentrations (Powell et al., 1980; Crowley et al., 1987). The inability to utilize hydroxamates could be considered as a negative fitness factor since hydroxamate siderophores are found in significant amounts in natural soils (Powell et al., 1980).

The successful performance of rhizobial inoculant strains depends upon their capability to outcompete the indigenous soil bacteria, survive and propagate, and enters into effective symbiosis with the host plant. The strains which fail to survive under soil conditions are most of the times ineffective in enhancing legume productivity because vast majority of nodules formed are not by the inoculated strain, but by indigenous rhizobia in the soil (Miller and May 1991; Streeter 1994). Thus construction of genetically engineered inoculum strains of *Rhizobium* with an increased ability to survive under soil conditions, and hence compete for nodule occupancy was considered an amicable approach to address the present problem. Most of the rhizobial biofertilizer strains are poor rhizospheric colonizers due to their inability to compete with the indigenous soil microflora for nutrients and one of the major concerns to nodulating rhizobia is the iron limitation in soil because of the iron rich enzymes involved in nitrogen fixation (Verma and Long, 1983). Utilization of foreign siderophores is considered to be an important mechanism to attain iron sufficiency. Pseudomonads which are known for their rhizospheric stability have diverse iron uptake systems and multiple receptor genes have been detected in their genomes (Dean and Poole 1993; Ankenbauer and Quan 1994). In contrast to this, TonB dependent siderophore receptors were very few in the genomes of members of rhizobiales; 3 were present in *R. etli*,

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3 in *Mesorhizobium* sp. BNC1, two in *Mesorhizobium loti*, 2 in *Ag. tumefaciens* and 2 in *S. meliloti* (Joshi, F. R., PhD thesis, M.S.U 2006). This suggested that by increasing the number of outer membrane siderophore receptors rhizobial strains could be made more efficient with respect to iron acquisition, and hence colonizing the rhizosphere. Most of the hydroxamate siderophores present in soil are of the ferrichrome-type. Because ferrichrome is synthesized by a variety of soil fungi, it is likely iron source in the rhizosphere where hydroxamate concentrations have been estimated to be as high as 10μM (Crowley, et al. 1987) and ferrichrome is found in nanomolar concentrations, as estimated by physicochemical (Holmstrom et al., 2004) as well as bioassay methods (Powell et al., 1983). As majority of soil bacteria are good utilizers of iron bound to hydroxamates (Jurkevitch et al., 1992), thus the rhizobia studied here would be at a competitive disadvantage when residing free in soils. It was therefore pertinent to engineer these strains with a ferrichrome receptor to increase their iron acquisition property and hence survival.

The present work deals with cloning of the ferrichrome receptor gene in rhizobial inoculant strains and understanding the effect of ferrichrome utilization, on rhizobial growth and survivability under conditions wherein, ferrichrome was made available by other producer species. This objective was achieved by heterologously expressing the *E. coli fhuA* gene in *C. cajan* rhizobia. Since the lac promoter provides a good constitutive expression system in rhizobia (Labes et al., 1990) the expression of *fhuA* was engineered under the control of this promoter. The expression of the cloned *fhuA* was first confirmed in *E. coli* by the rescue of the phenotype of *ΔfhuA* mutant and was subsequently introduced into the rhizobial strains and its expression monitored. The expression of *E. coli fhuA* in rhizobial strains imparted them the associated phenotypes viz. the ability to utilize iron complexed with ferrichrome and sensitivity to albomycin.

The ferri-ferrichrome uptake system found in *E. coli* acts in concert and is made up of four protein complement, the FhuA (OM receptor), FhuB (inner membrane permease), FhuC (ATPase) and FhuD (periplasmic binding protein) (Coulton et al. 1987). This system is known as the Fhu-Periplasmic Binding protein dependent Transport system (Fhu-PBT), where the periplasmic binding protein FhuD is responsible for directing the Fe$^{3+}$-ferrichrome
complex to FhuBC protein complex for its transport from the periplasm to the cytoplasm. An attempt to find homologs of Fhu operon in the entire genome of _R. etli_ revealed 3 FhuB homologs, 1 homolog of FhuD and 46 homologs of FhuC in the complete genome of _R. etli_ (Joshi, F.R., PhD thesis, M.S.U 2006). Among the rhizobia, _R. leguminosarum_ biovar viciae has been shown to have a Fhu-PBT type system for the uptake for the trihydroxamate siderophore vicibactin (Stevens, 1999). We failed to detect any FhuA homologs in _R. etli_, which correlated well with the ferrichrome non utilizing phenotype of _Rhizobium_ sp. In our studies it was seen that the expression of the outer membrane receptor alone enabled the rhizobia to take up ferrichrome which implied that additional transport activities involving periplasmic and inner-membrane bound proteins were present in the strains and were perhaps part of uptake machinery for some other unknown siderophore but were being recruited for ferrichrome utilization.

Brickman and Armstrong (1999) in a study dealing with the _fauA_ gene encoding the receptor for alcaligin, a siderophore produced by _Bordetella_ species found that the incorporation of _fauA_ gene alone could confer upon a siderophore deficient strain of _P. aeruginosa_ the ability to utilize ferric alcaligin. These observations reinstate the belief that among all the ligand-protein interactions of members of the bacterial iron-acquisition system, the binding of ferri-siderophores to the outer membrane receptor proteins is the most specific (Guerinot, 1994). For instance, in _E. coli_ separate outer membrane receptors transport ferric iron bound to aerobactin, ferrichrome, rhodotorulic acid and ferrioxamine, yet all use a common set of periplasmic and inner membrane components. Genome sequences of bacteria contain numerous putative ferri-siderophore receptor genes (Cornelis and Matthijs, 2002; Poole and McKay, 2003) but do not contain equivalent copies of the genes for periplasmic and cytoplasmic membrane-bound proteins. Our results provide evidence that engineering rhizobial strains by incorporating genes for multiple iron-siderophore receptors has potential in increasing the suite of ferric-siderophores that they can utilize.

The growth stimulation of only rhizobial transformant IC3123::pGR1 and not the parent IC3123 was observed when pure ferrichrome (15μM) was exogenously supplied. This substantiates that the rhizobial transformants are at a competitive advantage relative to the...
untransformed strains under conditions when iron is available only as bound to ferrichrome. Similar observation was made when ferrichrome-producing organism *U. maydis* was co-inoculated with the rhizobia. When pure ferrichrome was supplied externally it was observed that the growth rate of rhizobial transformants was somewhat lower than the parent strain. This may be attributed to metabolic load imposed by the presence of plasmid since it is well-documented that expression of plasmid encoded marker genes or inserted foreign genes impose metabolic burden on the organism (Glick, 1995a). Comparison of the growth rates of rhizobia bearing the vector plasmid and those harboring pGR1 would help discern whether the growth disadvantage is due to marker gene expression or *fhuA* expression. It is puzzling why a similar observation was not seen when the rhizobial transformants were co-inoculated with *U. maydis*. It may be speculated that this could be due to the production of some factors by *U. maydis* that helped the rhizobia to retain their normal growth profile. Several other studies have shown that utilization of heterologous siderophores provides growth advantage to rhizospheric bacteria Raaijmakers et al. (1995) introduced the siderophore receptor for ferric pseudobactin 358 into *P. fluorescens* WCS374, resulting in a strain which was more competitive than the WCS374 parental strain for colonization of the radish rhizosphere when co-inoculated with pseudobactin producing isolate *P. putida* WCS358. Siderophore producing *Penicillium chrysogenum* and *P. aeruginosa* significantly enhanced nodulation of mung-bean and nitrogen fixation by *Bradyrhizobium* species that could cross-utilize the *Penicillium chrysogenum* and *P. aeruginosa* siderophores (Mahmoud and Abd-Alla, 2001).

Pot inoculation studies were performed in order to compare the nodulation by the parent and the transformed strains in soil conditions and subsequent effect on plant growth. The transformants showed about 1.5-2.0 fold increase in nodule number per plant. The transformants clearly benefited the plant also in terms of shoot fresh weight and increased chlorophyll content. There are studies indicating a close correlation between leaf chlorophyll content and leaf N content (Henriksson and Pearson, 1981). Increased chlorophyll levels of the plant leaves inoculated with the transformants indicates efficient nitrogen fixation occurring in the nodules. Rhizospheric colonization and survival also increased with IC3123::pGR1 and ST1::pGR1 as compared to their parent under both autoclaved and
unautoclaved conditions. It is demonstrated that increased survival of the strains does improve their nodule occupancy on *C. cajan* which in turn has positive effects on plant growth. Transformants co-inoculated with *U. maydis* did show better survival and hence increased nodule occupancy, and were able to support plant growth better as compared to the parent ST1 co-inoculated with *U. maydis*. Ferrichrome being the most commonly produced siderophore by most soil fungi enhances the levels of iron available to the transformed strains, leading to better survivability, rhizospheric colonization, and nodule ability. Thus, the introduction of plasmid containing the ferrichrome uptake gene *fhuA* gene into *Rhizobium sp.* has been shown to have positive consequences on legume growth.

Benson, et al (2005) identified FegA as a ferrichrome receptor in *B. japonicum* 61A152. They showed that in addition to the FegA receptor, FegB was necessary for *B. japonicum* to utilize ferrichrome. They reported that in their experiments the *fegAB* mutant showed a strong phenotype in planta. In all assays *fegB* mutant was indistinguishable from the wild type. Thus it was shown that FegA was required to form a functional symbiosis. Since it is not possible that ferrichrome is made available to the nodulating strain *in planta*, the authors proposed that probably FegA protein might also act as receptor for some other compounds whose uptake might be critical in establishing an effective symbiosis. The studies reported here are also in favor of this hypothesis and additionally also suggest the role of ferrichrome-utilization ability in competitive survival in soil.