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Enhanced Survival and Nodule Occupancy of Pigeon pea Nodulating *Rhizobium* sp. ST1 expressing *fegA* Gene of *Bradyrhizobium japonicum* 61A152

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Abstract: Problem statement: Rhizobial isolates belonging to genera (*Rhizobium* sp. and *Mesorhizobium* sp.) in our laboratory produced only catecholate type of siderophores. Although FhuA and FegA (ferrichrome receptors) homologs were found to be present in the sequenced genomes of few rhizobia (e.g., 1 in *R. etli* and 2 in *Mesorhizobium* sp. BNC1), laboratory isolates of the corresponding genera failed to utilize ferrichrome, a siderophore which is present in nanomolar concentrations in the soil. This inability was considered as a negative fitness factor with respect to rhizospheric colonization by these rhizobia. **Approach:** The 2.4 kb *fegA* gene (encoding ferrichrome receptor) was amplified along with its native promoter from *Bradyrhizobium japonicum* 61A152 and cloned in a broad host range plasmid vector pUCPM18. The plasmid construct pFJ was transferred by conjugation into *Rhizobium* sp. ST1 to give transconjugant ST1pFJ12. The consequence of FegA expression on the transconjugant was tested under lab and soil conditions, using physiological experiments. **Results:** Ability of the transconjugant ST1pFJ12 to utilize ferrichrome and expression of a 79 kD protein band on the outer membrane of the transconjugant confirmed FegA expression. Transconjugant ST1pFJ12 exhibited increased growth rate as compared to the parent strain ST1, in minimal media containing ferrichrome as the sole iron source, confirming the positive effect of FegA expression. Inoculation of pigeon pea seedlings with transconjugant ST1pFJ12 led to a marked increase in plant growth parameters as compared to plants inoculated with the parent strain ST1, the effect being more pronounced when *Ustilago maydis*, a ferrichrome producer was co-inoculated in the systems. Nodule occupancy on pigeon pea plant when inoculated with the transconjugant ST1pFJ12 alone was 57% which increased to 66% when co-inoculated with *U. maydis* as compared with 37 and 30% respectively, seen with parental strain ST1 inoculation. **Conclusion:** The clear increase in nodule occupancy and higher rhizospheric colonization by the *fegA* transconjugants, presented in this study together with the previous research reported from our laboratory, led us to conclude that ferrichrome utilization ability played an important role in the rhizospheric colonization of the bioinoculant strains. Testing the ability to utilize hydroxamate siderophores therefore, holds prime importance in selecting an efficient biofertilizer strain.

Key words: Ferrichrome, rhizobia, rhizospheric colonization, cross-utilization, nodule occupancy

INTRODUCTION

Although iron is the fourth most abundant element on the Earth, it is mainly present in its oxidized state, having a solubility of 10^{-18}M at biological pH[1], which is much less than what is required by most soil microorganisms. The solubility further decreases 1000 fold with increase in pH by 1 unit, due to its tendency to form iron-hydroxides polymers in aqueous environments[1]. Microorganisms, including rhizobia, employ various mechanisms to acquire this essential nutrient, which includes production of iron chelating molecules known as siderophores, which bind Fe^{+3} with a very high affinity and form ferrisiderophore complexes. These ferrisiderophore complexes are taken inside the cells, aided by multi-component iron uptake systems, comprising of a high-affinity outer membrane receptor, which is specific to the ferrisiderophore ligand, in association with broadly specific periplasmic binding proteins and inner membrane ATPases.

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Rhizobia are amended into agricultural soils, but despite their efficient nitrogen fixing potential, most of the times they fail to increase plant yields, which is attributed to their inefficiency to successfully colonize the rhizosphere, iron availability being one of the limiting factors for rhizospheric colonization[6,7].

Rhizobia are known to produce a wide variety of siderophores; *Rhizobium meliloti* DM4 and *Sinorhizobium meliloti* produce rhizobactin[8-10], *Rhizobium leguminosarum* bv. *viciae* MNF7101 and WSM710 produce vicibactin[11], catecholate siderophores viz., salicylic acid and dihydroxybenzoic acid are produced by *R. ciceri* isolates from chick pea nodules[12] whereas uncharacterized catecholates are produced by rhizobia from the cowpea group[13,14]. In alkaline soils of the mid-western United States *B. japonicum* serotype 135, a siderophore producer, dominates over serotype 123, a non-siderophore producer[15]. In addition to that, 54% of *S. meliloti* strains isolated from alfalfa nodules from a high pH soil were siderophore producers, whereas those from a soil of lower pH, the proportion of siderophore-producing strains fell to 18%[16]. The above facts underline the importance of iron acquisition systems in rhizobial populations.

Fluorescent pseudomonads are most studied Plant Growth Promoting Rhizobacteria (PGPR) and one of the traits contributing to their PGPR status is not only their ability to produce large number of siderophores but also their ability to utilize equal numbers of heterologous siderophores (siderophores produced by other organisms) via various TonB dependent siderophore receptors. Studies report 32 putative siderophore receptors in *P. aeruginosa*[17,18], 29 in *P. putida*, 27 in *P. fluorescens* and 23 in *P. syringae*[19]. Amongst rhizobia *B. japonicum* 61A152 is reported to be a successful bioinoculant strain for soybean crop[22]. *Bradyrhizobium japonicum* 61A152 produces citrate as the only siderophore[20], but also can internalize iron complexed with ferrichrome and rhodotorulic acid, the hydroxamate siderophores produced by many soil fungi[21]. This is attributed to the presence of *fegA* gene, encoding ferrichrome receptor, in this organism. The concentration of hydroxamate type siderophore in soil is reported to be as high as 10 μM[23], ferrichrome, constituting the major fraction amongst these[24].

Most of the rhizobial isolates and other nodule bacteria tested in our laboratory produced mainly catecholate type of siderophores and failed to utilize iron complexed with ferrichrome, a hydroxamate siderophore known to be present in abundance in the soil[26-29]. Many studies indicate that efficient utilization of hydroxamate siderophores by rhizobia is a positive fitness factor with respect to its soil survival[22,45]. The objective of the present study therefore was to impart upon these rhizobia ferrichrome utilization ability so as to increase their competitive survival in rhizosphere.

Recent study from our laboratory has shown that the expression of *Bradyrhizobium japonicum* 61A152 *fegA* gene in peanut rhizobia, not only supports the growth of the *fegA* transformants under iron limited laboratory conditions, but also increases its survivability under natural soil conditions, which led to higher nodulation on peanut plant[29]. Similar study regarding the expression of *B. japonicum fegA* in pigeon pea rhizobia, *Rhizobium* sp. ST1, presented in this investigation would, not only substantiate the earlier studies, but also would allow us to generalise the previous observation that hydroxamate siderophore utilization ability confers upon a strain competitive survival and therefore can be considered as an important criteria while selecting an efficient bioinoculant strain. Studies have also been extended towards carrying out an *in silico* investigation of the occurrence of ferrichrome receptor genes in sequenced genomes of various rhizobia to substantiate our findings.

MATERIALS AND METHODS

Bacterial strains, plasmids and growth conditions: The bacterial strains and plasmids used in this study are as in Table 1. *Rhizobium* sp. ST1 (16SrRNA gene sequence Genbank Accession number DQ632608), a nitrogen fixing nodule symbiont of pigeon pea (*Cajanus cajan*) is a lab isolate; *S. meliloti* IC3169 (*C. cajan*); *Mesorhizobium* sp. GN25 (*Arachis hypogea*, 16SrRNA gene sequence Genbank Accession number DQ862066)[31] and *Bradyrhizobium* sp. NC92 (*A. hypogea*) were procured from Indian Agricultural Research Institute (IARI), New Delhi, India; *B. japonicum* 61A152 was a kind gift from Guerinot, M.L., Dartmouth college, Hanover, USA[32]. All the above strains were maintained routinely on YEM medium (1% mannitol, 0.1% yeast extract, 0.02% $MgSO_4.7H_2O$, 0.01% NaCl, 0.05% K_2HPO_4). Ashby's Mannitol (AM) broth (1% mannitol, 0.2% sodium glutamate, 0.02% $MgSO_4.7H_2O$, 0.01% NaCl, 0.05% K_2HPO_4) deferrated using 0.25% 8-hydroxyquinoline in chloroform was used as iron limited media for all the rhizobial strains and ST1pFJ12, *fegA* transconjugant of *Rhizobium* sp. ST1. Iron supplemented medium was prepared by adding 100 μM $FeCl_3$ to the above medium. The medium as described by Winkelmann was used for siderophore production by *U. maydis* and *Rhodotorula mucilaginosa* MTCC 850[31] *P. fluorescens* ATCC13525, *P. aeruginosa* MTCC2453 and *P. putida* KT2440 were from our laboratory collection;

Table 1: Bacterial strains and plasmids used in this study

Bacterial/fungal strains or plasmids	Relevant characteristics	Source or reference
Rhizobial strains:		
Rhizobium sp. ST1	Nitrogen fixing pigeon pea (*Cajanus cajan*) nodule symbiont, 16SrRNA gene sequence accession number DQ632608, Rifampicin^R	Laboratory collection
Mesorhizobium sp. GN25	Peanut (*Arachis hypogea)* nodule symbiont, 16SrRNA gene sequence accession number DQ862066	IARI, New Delhi, India
Sinorhizobium sp. IC3169	*Sinorhizobium* sp., pigeon pea biofertilizer strain	IARI, New Delhi, India
Bradyrhizobium sp. NC92	*Bradyrhizobium* sp. peanut biofertilizer strain	IARI, New Delhi, India.
Bradyrhizobium japonicum 61A152	Nitrogen-fixing *Glycine max* (soybean) symbiont	[30]
ST1pFJ12	ST1 carrying pFJ	Present study
***Escherichia coli*:**		
DH5α	*hsdR17 endA1 thi-1 gyrA96 relA1 supE44* Δ*lac U169* (φ*80dlacZ*Δ*M15*)	[38]
S17.1	*hsdR pro recA* containing RP-4-2-Tc:: Mu integration into chromosome	[39]
S17.1pFJ	*E. coli* S17.1 carrying pFJ	Present study
MB97pFJ	MB97Δ*fhuA* carrying pFJ	This study
***Pseudomonas* strains:**		
P. fluorescens ATCC13525	Used as a source of heterologous siderophores	Laboratory collection
P. aeruginosa MTCC2453	Used as a source of heterologous siderophores	Laboratory collection
P. putida KT2440	Used as a source of heterologous siderophores	Laboratory collection
Plasmids		
pTZ57R-T	Used for cloning the *fegA* amplicon	[MBI Fermentas]
PTZfegA	*fegA* amplicon cloned in pTZ57R-T	Present study
pUCPM18-Gm	*Escherichia-Pseudomonas* shuttle vector pUCP19, containing the *mob* fragment	[37]
pFJ	pUCPM18-Gm with *fegA* cloned in XbaI-BamHI site	Present study

positive for siderophore production and their supernatants at the end of 48 h of growth under iron limiting condition was used as sources of siderophores.

Whenever required, the following concentration of antibiotics was supplemented in the media-for rhizobia: Ampicillin (Amp 150 μg mL^{-1}); rifampicin (Rif 50 μg mL^{-1}) and gentamycin (Gm 80 μg mL^{-1}) and for *E. coli*: Ampicillin (Amp 100 μg mL^{-1}); gentamycin (Gm 40 μg mL^{-1}).

Siderophore production and cross-utilization bioassay: Siderophore production was induced in all the rhizobial strains by growing them in deferrated AMB for 48 h and the supernatants were tested for the presence of catecholate[32] and hydroxamate[33] siderophores, using 2,3-dihydroxybenzoic acid and hydroxylamine hydrochloride respectively, as standards. Siderophore cross-utilization bioassay was performed exactly as described by[29].

Sequence analysis: Sequences from various organisms annotated as FhuA, FhuB, FhuC and FhuD respectively, were collected from the SWISSPROT/TrEMBL[34] database. The sequences for each group were aligned using MUSCLE[35]. The HMM-build_mw program was used to construct profile-HMMs from the alignments and the HMM-search program (E-value cut-off of 0.1) was used to scan the protein sequences in the genomes of *Rhizobium etli* CFN42, *Sinorhizobium meliloti* 1021, *Mesorhizobium loti* MAFF303099, *Mesorhizobium* sp. BNC1, *Rhizobium leguminosarum* bv *viciae* 3841 and *B. japonicum* USDA110 along with their plasmid sequences. As the number of sequences of FegA and FegB were insufficient to construct HMMs, BLAST searches against the genomes were used to identify homologs[36] (E-value cut-off of 0.1).

Primer designing and PCR amplification of *fegA* and construction of plasmid pFJ: The *fegA* amplicon was cloned in pGEM-T vector (MBI Fermentas) and subcloned subsequently in pUCPM18-Gm[37] exactly as described in[29].

DNA manipulations: Genomic DNA isolation was done as described in[38]. Plasmid DNA isolations (from *E. coli* and rhizobia), DNA ligations, transformation of *E. coli* with plasmid DNA were also performed using standard procedures[38]. Restriction endonuclease digestions were performed according to manufacturer's instructions (MBI Fermentas). pFJ was transferred from S17.1 containing pFJ to *Rhizobium* sp. ST1 by patch mating method as described by[39].

Growth assays: Initial inoculum was prepared by growing the cultures in YEM medium and 1% of 1.0 OD culture was inoculated in iron limited media. Pure ferrichrome was exogenously added to a final

concentration of 15 μM. Growth was measured as optical density at 600 nm every 6 h post inoculation.

SDS-PAGE analysis of Outer Membrane Proteins (OMPs): OMP extraction was done by the method of[40] with minor modifications as described in[29] for rhizobial cultures.

Plant studies: Agricultural soil was collected from Anand agricultural university, Model farm, Vadodara, Gujarat. Analysis of soil parameters was done at Gujarat State Fertilizer Corporation, Vadodara, Gujarat, India. The soil was alkaline (pH 7.8, with electrical conductivity of 0.27 mmho cm^{-1} and organic C content of 0.57 g kg^{-1}. The total iron concentration was 14.66 ppm, as estimated by atomic absorption spectroscopy. The total siderophore concentration was estimated to be 3.8 μM using CAS solution[41,42]. Pigeon pea [BDN-2, Gujarat, India] seeds were surface sterilized, coated with ST1 and ST1pFJ12 and the pot experiments were set up in the same way as done in[29], but only under natural (un-autoclaved) soil conditions. The pots were incubated under natural day-light conditions and at the end of 30 days were checked for various parameters like shoot fresh weight, root fresh weight, chlorophyll content of the leaves and nodule density. Plants without inoculation of either rhizobial and/or fungal cultures were used as controls. The count of the parent strain ST1 (RifR) and transconjugant (RifR, AmpR, GmR) colonizing the rhizosphere was determined by plating appropriate dilutions of rhizospheric soil suspension {1:1 soil (g): Sterile saline (mL)} on YEM plates containing the appropriate antibiotics. Nodule occupancy by inoculated organisms was determined exactly as in[29].

Statistical analysis: The statistical analysis of the results obtained was done by one way ANOVA (analysis of variance) using the web-trial version of SigmaStat 3.5. Null hypothesis was set on mean difference equal to 0 and Alpha at 0.05. The difference between all the comparisons made is significant at 95% confidence interval.

RESULTS

Pseudomonads are known for their rhizospheric stability and one of the factors contributing to this is the presence of diverse iron uptake systems. About 32 putative siderophore receptors in *P. aeruginosa*[17,18], 29 in *P. putida*, 27 in *P. fluorescens* and 23 in *P. syringae*[19] are reported. Analysis of protein sequences of complete genomes of *Pseudomonas* species using HMMER profiles revealed the presence of 45 TonB dependent siderophore receptors in the genome of *P. fluorescens*, 31 in *P. putida* and 36 in *P. aeruginosa*. In contrast, a complete genome wide search in a few members of rhizobiales revealed a visible scarcity of TonB dependent siderophore receptors; 3 were present in *R. etli*, 3 in *Mesorhizobium* sp. BNC1, 2 in *Mesorhizobium loti*, 2 in *Sinorhizobium meliloti* and 8 in *Bradyrhizobium japonicum* (Table 2) Relatively high number of TonB dependent receptors present in *Bradyrhizobium* sp. amongst rhizobiales could be attributed for their high rhizospheric competence and hence their reported success as commercial biofertilizers for soybean crops[22]. This led us to conceptualize that increasing the repertoire of outer membrane siderophore receptors could make our rhizobial isolates more efficient with respect to iron acquisition and hence colonizing the rhizosphere. Laboratory rhizobial isolates *Rhizobium* sp. ST1, *Mesorhizobium* sp. GN25, *Sinorhizobium* sp. IC3169 were found to produce only catecholate type of siderophores (Table 3) and failed to utilize hydroxamate siderophores, mainly ferrichrome and rhodotorulic acid (Table 3). Since rhizobia generally lack the ability to produce and utilize hydroxamates[26-29], it is hypothesized that imparting upon them the ability to utilize hydroxamate siderophores would lead to increase in their rhizosperic competence.

Table 2: *In silico* identification of TonB dependent siderophore receptors in complete genomes of pseudomonads and rhizobia

Organism whose sequence was taken	No. of TonB dependent receptors detected
P. aeruginosa	36
P. fluorescens	45
P. putida	31
Mesorhizobium loti	1
Mesorhizobium sp. BNC1	3
Rhizobium etli CFN42	3
Sinorhizobium meliloti	2
R. leguminosarum	3
Bradyrhizobium japonicum USDA110	8

Table 3: Siderophore production and cross-utilization by the rhizobial isolates

	Catecholate siderophore production	Siderophores tested for cross-utilization						
Isolate	(μg mL^{-1})	FC	RA	Desf	PF	PA	PP	Fe-cit
61A152	-	+	+	+	-	-	+	+
ST1	15.32	-	-	-	-	-	+	+
IC3169	2.21	-	-	-	-	-	+	+
GN25	2.93	-	-	+	-	-	+	+
NC92	3.02	+	+	+	-	-	-	+

(+): indicates growth and (-): indicates no growth around the siderophore containing wells; FC: ferrichrome, RA: Rhodotorulic acid, Desf: desferal, PF: *Pseudomonas fluorescens* culture supernatant, PA: *P. aeruginosa* culture supernatant, PP: *P. putida* culture supernatant

Table 4: Identification of ferri-ferrichrome uptake machinery (FegA, FhuA, FhuB, FhuC and FhuD) homologs from sequenced genomes of rhizobia

	R.etl	M.BNC1	M.lot	R.leg	S.mel	B.jap
FegA	1 (35%)	1 (32%)	0	2 (33 and35%)	2 (42 and34%)	2 (84 and 40%)
FhuA	1	2	0	2	4	4
FhuB	5	4	2	5	5	1
FhuC	110	74	114	153	113	87
FhuD	1	1	1	1	0	0

The numbers in the table indicate the number of hits obtained in the genomes with the profile-HMMs of FhuA, FhuB, FhuC and FhuD. The numbers in parenthesis indicate the percentage identity of the hit obtained with the FegA sequence using BLASTp; R.etl: *Rhizobium etli* CFN42 along with plasmids p42a, p42b, p42c, p42d, p42e, p42f; M.BNC1: *Mesorhizobium* sp. BNC1 along with plasmids pL1, pL2, pL3; M.lot: *sorhizobium loti* along with plasmids pMLa, pMLb; R.leg: *R. leguminosarum biovar viciae* 3841 along with plasmids pRL7, pRL8, pRL9, pRL10, pRL11, pRL12; S.mel: *Sinorhizobium meliloti* 1021 along with plasmids pSymA, pSymB; B.jap: *Bradyrhizobium japonicum* USDA110 genome.

Ferrichrome constitutes one of the major hydroxamate siderophores in the soil[24]. *In silico* studies were performed to search specifically the ferri-ferrichrome uptake machinery homologs (FegAB of *B. japonicum* 61A152 and FhuABCD of *E. coli*) in sequenced genomes of rhizobia. Based on the percent identity of the hits obtained in BLASTp using FegA as the query sequence, FegA homologs of significant identity were detected in *S. meliloti* and *B. japonicum*, but not in *Rhizobium* sp. and *Mesorhizobium* sp. (Table 4). We failed to detect FegB homologs in any of the rhizobial genomes. Two homologs of FhuA were present in *Mesorhizobium* sp. BNC1 (GI: 110632699 and GI: 110633793), 2 in *R. leguminosarum* biovar *viciae* 3841 (GI: pRL120322, GI: pRL100325) and 1 in *R. etli* CFN42 (GI: 86361199). In addition to that enough FhuBCD homologs were present in them to partner the FhuA homologs. However, FhuBCD homologs were detected in *Mesorhizobium loti* MAFF303099 inspite of the absence of FhuA homologs in its genome.

Four FhuA homologs were detected each in *S. meliloti* (GI: 15965976, GI: 15966022, GI: 16263416, GI: 15963968) and *B. japonicum* USDA110 (GI: 27379615, GI: 27380031, GI: 27383079, GI: 27379015) genomes. It was also of importance that FhuD homologs were detected in genomes of *Rhizobium* sp. and *Mesorhizobium* sp. studied, while they were absent in *B. japonicum* and *S. meliloti*. The rhizobial strain under present study, *Rhizobium* sp. ST1 failed to utilize hydroxamate siderophores, ferrichrome and rhodotorulic acid (produced by many fungi) and desferal (produced by actinomycetes) as iron sources (Table 3) and hence was selected for the introduction of the *fegA* gene encoding ferrichrome receptor from *B. japonicum* 61A152 and to check the effect of its expression on rhizospheric colonization by the strain.

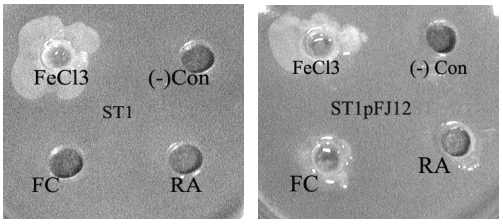

Fig. 1: Ferrichrome (FC) utilization by the transconjugant ST1pFJ12. The *fegA* transconjugant ST1pFJ12 exhibits a zone of growth exhibition around the FC (30 μg) containing well in contrast to the parent strain *Rhizobium* sp. ST1

A 2.4 kb *fegA* gene was amplified from *B. japonicum* 61A152 using *fegA* gene specific primers (data not shown) and confirmed by sequencing. No *fegA* amplification was obtained with genomic DNAs of *Rhizobium* sp. ST1, *Sinorhizobium meliloti* IC3169, *Mesorhizobium* sp. GN25 and *Bradyrhizobium* sp. NC92 used as templates (data not shown). The *B. japonicum* 61A152 *fegA* amplicon was cloned into pUCPM18-Gm vector as per[29] to obtain construct pFJ. *fegA* (pFJ) was subsequently transformed into *E. coli* S17.1 (S17.1pFJ) and by patch conjugation with S17.1pFJ *fegA* was mobilized into *Rhizobium* sp. ST1. The transconjugants were screened on Rif-Amp-Gm plates. The presence of *fegA* in the transconjugant ST1pFJ12 was confirmed by restriction digestion analysis of the plasmid isolated and by colony PCR of the *fegA* gene (data not shown). Transconjugant ST1pFJ12 was tested for the cross-utilization of all the siderophores previously tested and as opposed to the parent strain ST1, transconjugant ST1pFJ12 could utilize the siderophore ferrichrome (Fig. 1). A 79 kDa iron regulated outer membrane protein was found to be present in the outer membrane protein preparation of ST1pFJ12 which correlated with the FegA protein of *B. japonicum* 61A152[30] and was absent in the parent strain ST1 (Fig. 2). This confirmed the expression of FegA in the transconjugant ST1pFJ12. Significant increase in growth of the transconjugant ST1pFJ12 was observed in iron limited conditions, in the presence of pure ferrichrome (15 μM) in comparison to its absence (Fig. 3b), while no such growth stimulation due to ferrichrome was seen with parent strain ST1 (Fig. 3a). It was however observed that the presence of ferrichrome partially inhibited the growth of parent ST1 (Fig. 3a).

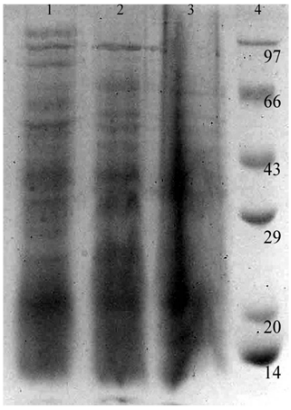

Fig. 2: Outer membrane profiles to check expression of FegA in *fegA* transconjugant of *Rhizobium* sp. ST1. (1): *B. japonicum* 61A151 Fe (-) condition. (2): transconjugant ST1pFJ12 Fe (-) condition (3): parent strain ST1 Fe(-) condition

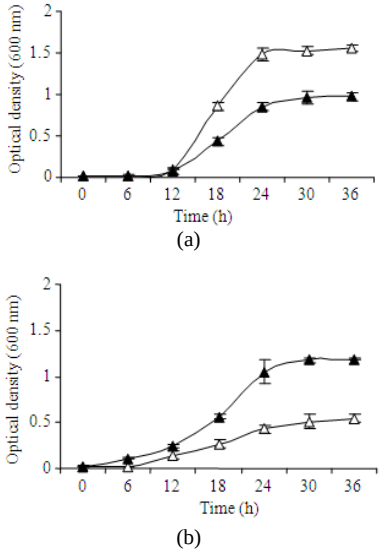

Fig. 3: Growth of (a): ST1 and (b): *fegA* transconjugant ST1pFJ12 in the presence (filled triangles) and absence (open triangles) of externally supplemented ferrichrome (15 µM). The values represent mean of three independent experiments, vertical bars indicate standard deviation

Pot inoculation studies were performed under natural soil conditions in order to check the potential of the transconjugant ST1pFJ12 for rhizospheric colonization and nodulation on pigeon pea, the nodulating host of ST1. Pigeon pea seedlings inoculated with ST1pFJ12 produced significant increase in shoot

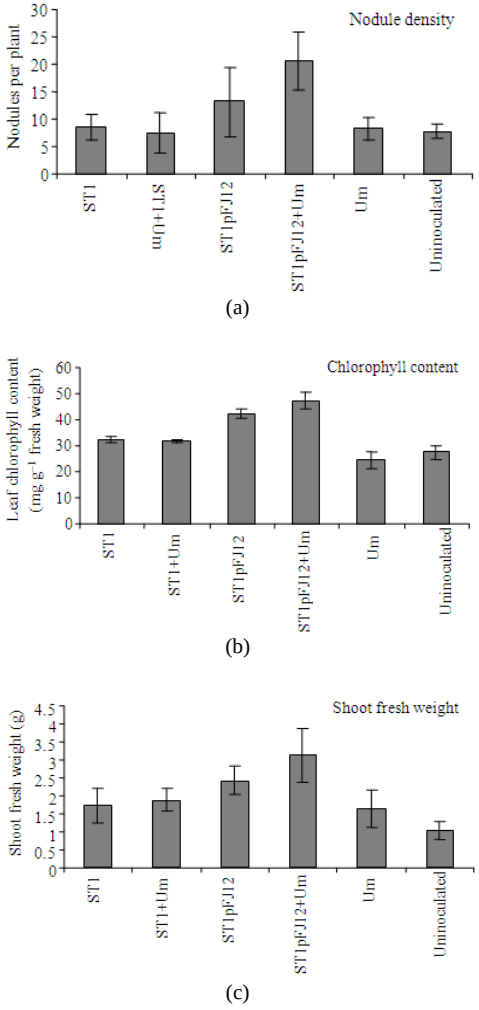

Fig. 4: Influence of *fegA* transconjugant ST1pFJ12 inoculation on pigeon pea plant parameters as compared to parent strain *Rhizobium* sp. ST1 inoculation in natural soil condition, in the presence and absence of ferrichrome producing fungus *Ustilago maydis*. Plants were harvested 30 days post inoculation and were assayed for average chlorophyll content of leaves, average shoot fresh weight and average nodule density

and root fresh weight, nodule density ($p<0.001$) and chlorophyll content ($p<0.05$) of the leaves as compared to ST1 inoculation or uninoculated control plants, the difference being more prominent when *U. maydis* was co-inoculated in the systems (Fig. 4). This increase in plant parameters correlated with the counts of the inoculated bacteria in the rhizospheric soil which were estimated at start of the experiment and at the end of 30 days post-inoculation on selective antibiotic plates.

Table 5: Nodule occupancy by *Rhizobium* sp. ST1 and transconjugant ST1pFJ12 in the presence and absence of *Ustilago maydis* inoculated under natural soil conditions

Condition	Total No. of nodules	No. of nodule isolates growing on antibiotic plates	Effective nodulation by inoculated strains	Nodule occupancy (%)
ST1	69	31 (Rif)	26	37
ST1+Um	71	33 (Rif)	28	30
ST1pFJ12	84	58 (Rif/Amp/Gm)	51	57
ST1pFJ12+Um	112	82 (Rif/Amp/Gm)	75	66
Um	49	6 (Rif); 6 (Amp/Gm)		
Uninoculated	46	4 (Rif); 8 (Amp/Gm)		

Starting with approximately 8.6×10^4 cfu g^{-1} rhizospheric soil, the ST1 population decreased to 2.1×10^3 and 1.9×10^2 cfu g^{-1} rhizospheric soil in the absence and presence of *U. maydis*, respectively. In contrast to this ST1pFJ12 was able to increase their populations to 1.0×10^5 and 3.4×10^5, when inoculated alone and when co-inoculated with *U. maydis*, respectively, starting with an initial population of 7.9×10^4. The nodule occupancy, as calculated by scoring the pure cultures isolated from nodules on antibiotic marker plates, was found to be 57% and 37% on the plants inoculated with transconjugant ST1pFJ12 and parent strain ST1, respectively (Table 5). No significant difference in the fresh weight of pigeon pea plants was observed when *U. maydis* was co-inoculated with *Rhizobium* sp. ST1 ($p = 0.924$), but a slight decrease in nodule occupancy was observed (Table 5). However *U. maydis* when co-inoculated with transconjugant ST1pFJ12, showed increase in the nodule occupancy to 66%, with no significant effect on shoot fresh weight but an increase in the chlorophyll contents of the leaves. Thus a positive correlation between nodule occupancy and chlorophyll content of the leaves of pigeon pea was observed.

DISCUSSION

The plant rhizosphere is a dynamic environment where intense competitive interactions take place among microorganisms and many factors affect the microbial composition, the main among these being iron. The iron acquisition problem is more pronounced in rhizobia because nitrogen fixation involves iron rich proteins like nitrogenase, leghemoglobin and cytochromes, with nitrogenase and leghemoglobin constituting up to 12 and 30% of total protein in the bacterial and infected plant cells, respectively[43]. Earlier studies from our lab have demonstrated that *Arachis hypogea* and *Cajanus cajan* root nodule isolates of rhizobia are poor producers and utilizers of hydroxamate-type siderophores[26-29,47]. Recent study from our laboratory has shown that the expression of *Bradyrhizobium japonicum* 61A152 *fegA* gene in peanut rhizobia, not only supports the growth of the *fegA* transconjugants under iron limited laboratory conditions, but also increases its survivability under natural soil conditions, leading to higher nodulation on peanut plant[31]. Similar study presented here regarding the expression of *B. japonicum fegA* in pigeon pea rhizobia, *Rhizobium* sp. ST1, not only substantiates the earlier results, but also allows us to hypothesize that hydroxamate siderophore utilization can be considered as an important criteria while selecting a bioinoculant strain. In order to determine the relative importance of ferrichrome utilization, we have also carried out an *in silico* investigation of the occurrence of ferrichrome receptor proteins in sequenced genomes of various rhizobia.

Rhizobial strains investigated here were found to produce and utilize catechol-type siderophores, but were poor producers and utilizers of hydroxamate-type siderophores. In contrast to our observations, majority of the rhizobial inoculant strains for Australian pulse and legume crops from the Australian Inoculants Research and Control Service (AIRCS) are reported to be producers of either tri- or di-hydroxamate siderophores and are successfully used as commercial inoculants for pulse and legume pastures[45]. Field tests performed with *B. japonicum* 61A152, consistently gave high yields of soybean cultivars[22] and this led to the speculation that iron-scavenging property of *B. japonicum* 61A152 may be responsible for its competitive survival in the rhizosphere and hence better nodulation.

The *fegA* gene with its native promoter was cloned into pUCPM18-Gm (pFJ) and when transferred into *Mesorhizobium* sp. GN25[29] and *Rhizobium* sp. ST1 (present study), imparted the ability to utilize ferrichrome, implying that the ferrichrome transport through the outer membrane could be complemented by the inner membrane protein machinery already present in these organisms. A finding similar to ours, where expression of only the outer membrane receptor imparts siderophore utilization has been reported where the expression of *fauA* gene, encoding receptor for alcaligin siderophore, imparts alcaligin utilization to a *P. aeruginosa* strain deficient in alcaligin production[46] and introduction of *fhuA* gene brings about ferrichrome utilization in rhizobial strains deficient of ferrichrome production as well as utilization[47]. Growth stimulation of only the transconjugant ST1pFJ12 and not the parent strain ST1 in the presence of externally added ferrichrome, under iron limited growth conditions implies that, as opposed to the parent strain ST1, the transconjugant has a clear growth advantage. Hydroxamate type siderophores are

the main amongst the siderophores found in soil[23] most of it being of ferrichrome type, present in nanomolar concentrations[24]. *Rhizobium* sp. ST1 would therefore be at a competitive disadvantage when in direct competition with other rhizospheric organisms, most of them reported to be able to utilize iron bound to hydroxamates[25]. Present results provide evidence that engineering rhizobial strains with ferrichrome utilization ability add a competitive edge in an environment where ferri-ferrichrome is the main available source of iron.

It was surprising that we detected *Fhu*A homologs in genomes of *S. meliloti*, *Mesorhizobium* sp. BNCI and *R. etli* but the laboratory isolates of the corresponding genera failed to utilize ferrichrome. Possession of genetic elements for ferrichrome utilization system even differed among the two strains of mesorhizobia analyzed in this study. Rhizobiales are reported to have large genomes with disproportionate enrichment of high-affinity nutrient acquisition and secondary metabolism[50,51]. This genome plasticity is predicted to be of evolutionary advantage in soil environments where resources are scarce but diverse[51,52] and intraspecific genetic variation contributes to niche adaptation. Our results of inconsistency of ferrichrome cross-utilization, among members of the same genus, were justifiable in light of the above reports. In *Mesorhizobium* sp. BNC1, out of the 2 FhuA homologs identified by profile-HMM search, one was a TonB-dependent heme/hemoglobin receptor family protein (GI: 110633793) and FhuBCD homologs (GI: 110633795; GI: 110633796; GI: 110633797) were also found in close proximity, most probably organized to form an operon. The other FhuA homolog (GI: 110633699), encoded a TonB dependent siderophore receptor, was not associated with any of the FhuBCD homologs detected. *R. leguminosarum* biovar *Viciae* among the rhizobia, has been shown to have a Fhu PBT (Periplasmic Binding protein-dependent Transport) system for the uptake of trihydroxamate siderophore vicibactin[5]. In our analysis of *R. leguminosarum* genome, the iron-siderophore uptake machinery was present on plasmid pRL, different from other rhizobia, where the iron uptake machinery was present on the genome and as already reported[5] were clustered together as FhuABDC (GI: pRL100325, GI: pRL100326, GI: pRL100327 and GI: pRL100328). One FhuD homologue each was also present in genomes of *Rhizobium* sp. and *Mesorhizobium* sp. and a closer look at the genome sequence suggested the presence of Fhu-PBT type siderophore uptake system, associated with at least one FhuA homolog in *Rhizobium* and *Mesorhizobium* sp. The other FhuA homologs were not found to be associated with inner membrane machinery, similar to FhuE (rhodotorulic acid and coprogen receptor) and IutA (aerobactin receptor) of *E. coli*, which are also found in isolation[4]. These receptors work in association with FhuBCD (ferrichrome system) suggest that the transport of ferri-siderophores through the inner membrane is not as specific as that through the outer membrane. It is due to this reason that a notably less number of periplasmic and cytoplasmic membrane proteins are reported to be present in *Bradyrhizobium* sp. and *Pseudomonas* sp. against a relatively large number of outer membrane receptors reported[2]. Four homologs of FhuA in *B. japonicum* USDA110 (GI: 27379615; GI: 27380031; GI: 27383079; GI: 27379015) and one in *S. melitoti* (GI: 16263416) were detected. RhtX and FptX are members of a novel family of permeases for the transport of rhizobactin and ferricpyochelin respectively, through the inner membrane[3]. Though FegB does not share any sequence similarity to RhtX or FptX, it may be acting in a similar manner to transport ferrichrome across the inner membrane[2]. We failed to find homologs of FhuD in *B. japonicum* and *S. meliloti* in support to the above observations that transport of ferrisiderophores through the inner membrane in *S. meliloti* and *B. japonicum* is FhuD independent, dissimilar to the Fhu-PBT uptake system of *E. coli*. Because FhuC is a cytoplasmic ATPase and ATPases are highly conserved proteins, they were detected in very high numbers as compared to permease proteins which had unique sequences that made them specific for classes of siderophores or various other molecules which are also transported through ABC transporter permeases.

The successful performance of rhizobial inoculant strains depends upon their capability to out compete the indigenous soil bacteria, survive, propagate and enter into effective symbiosis with the host plant. Biofertilizer 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[6,7,44]. *Rhizobium* sp. ST1 was inhibited slightly in the presence of ferrichrome under lab conditions and also under natural soil conditions, which also gets reflected on its nodule occupancy on pigeon pea plants when co-inoculated with *U. maydis*. This might be because ST1 is unable to utilize ferrichrome and it could be argued here that the siderophore it itself produces is of a significantly lower affinity then ferrichrome[26]. Our results of plant inoculation state that expression of *fegA* into *Rhizobium* sp. ST1 increases the rhizospheric stability of the resultant transconjugant, which is evident by increase in

nodule occupancy on pigeon pea plants. It is demonstrated that having many nodules occupied by the inoculated rhizobial strains (ST1pFJ12) have positive effects on growth, nodulation and chlorophyll content of the plants and hence it is more likely that it would even affect legume yield. Previous reports have shown that co-inoculation of *B. japonicum* USDA110 with pseudomonads producing siderophores utilizable by the strain, enhances its nodulation frequency even in the presence of other competing bradyrhizobial strains[48]. Several rhizobial species have been reported to produce siderophores under culture conditions[10,11], but there is no evidence that these siderophores are made *in planta*. There are also evidences that mature N_2-fixing bacteroids of *R. leguminosarum* do not transcribe the vicibactin uptake receptor gene at detectable levels[49]. It is possible that FegA also does not have anything to do with iron uptake *in planta*[2]. Whether siderophore production or uptake efficiency increases the nitrogen fixation ability of nodule bacteria is not yet known, but their survival in the absence of their host definitely depends upon this characteristic.

CONCLUSION

Because ferrichrome is synthesized by a variety of soil fungi, it is likely to be the main iron source in the rhizosphere. Expression of *fegA* in *Rhizobium* sp. ST1, not only imparted upon the strain ability to utilize ferrichrome, but also increased its survivability in the soil, substantiating the hypothesis put forward. Co-inoculation of ferrichrome producing *U. maydis* did not have any antagonistic effect on plant growth, however transconjugant ST1pFJ12 co-inoculated with *U. maydis* do show better survival and hence increased nodule occupancy, as compared to parent ST1, co-inoculated with *U. maydis*. The results obtained here in association with other similar reports from our laboratory[26-29,47] support the fact that iron sufficiency of any organism largely depends on its ability to utilize the siderophore present in large concentrations in its vicinity[29,48]. Survival under iron limiting soil conditions is an important quality which every biofertilizer strain must possess. We therefore conclude that iron availability is one of the major factors determining rhizospheric colonization and therefore in order to select an efficient biofertilizer strain, its hydroxamate siderophore utilization property also holds a prime importance.

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