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Characterization of a Tn5-induced siderophore over-producing mutant of *Rhizobium* nodulating chickpea

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Rhizobium BICC 651, a chickpea (*Cicer arietinum* L.) nodulating strain, was mutagenised with transposon Tn5. The resultant mutant colony bank was screened for mutants on chrome azurol sulfone (CAS) plates for over-production of siderophore and, thus, two mutants were isolated. These mutants had higher level of siderophore in the culture filtrate than their parent strain under iron-limiting condition and were repressed for siderophore production in presence of iron (50 μm $FeCl_3$), as such were not considered as siderophore regulatory mutants. One of these mutants designated as B00l was found to be impaired in the uptake of ^{59}Fe-siderophore complex within the cell. A BamHI genomic DNA fragment adjacent to &e posterior end of Tn5 in the mutant was amplified by inverse PCR following self-ligation and was sequenced. Upon BLASTX search, part of the sequence of the fragment (265 bp) adjacent to Tn5 was found to have its highest homologs (90 %) to that of siderophore interacting protein of *Agrobacterium tumefaciens* CCNWGSO2S6 suggesting that Tn5 interrupted the gene that codes for a protein required for uptake of ferri-siderophore complex wifiin the cell. Symbiotic efficiency of the mutant was examined and was found to produce about 30 % higher number of nodules per plant as compared to its parent strain.

Key words : Chickpea nodulation, *Rhizobium*, siderophore over-producing mutants, Ferri-siderophore complex uptake.

INTRODUCTION

Biological processes input almost two-thirds of the fixed nitrogen in soil from the atnosphere in the world (Postgate, 1982).Among these processes legume-rhizobium symbiosis plays a crucial role. Legume-rhizobium symbiosis is iron dependent (Expert and Gill, l99l). Iron is required for successful infection and nodulation to occur, and for the synthesis of key proteins such as the leghaemoglobin, nitrogenase complex and numerous electron trapsport proteins to energize the nitrogenase system. Though it is the fourth most abundant element comprising 4.7 % of the earth's crust availability of iron in aerobic environment at biological pH is very low. Under such conditions iron tends to precipitate forming oxyhydroxide polymers of general composition FeOOH (Neilands and Leong, 1986). The solubility product constant for ferric hydroxide is about 10^{-38}. At neutral pH the free available iron is at a concentration of no more than 10^{17} M, which is far below that required for microbial growth (10^{-9}M). Microorganisms face the challenges of survival as well as to compete with other iron-requiring microorganisms in such an environment in the soil. Rhizobia face additional challenges when they enter into a nitrogen-fixing symbiosis with their host plants. They have increased need of iron from the host as microsymbionts to produce three times as many cytochromes as when they are free-living (Sangwan and O'Brian, 1992). To face these challenges many microorganisms including rhizobia produce a high-affinity iron acquisition system. The system involves the secretion of low-molecular-mass ferric-specific ligand termed siderophore which binds to available Fe^{3+} to form

ferri-siderophore complex. The complex is internalized by the organism for its iron rquirement. However, many organisms are also able to utilize the siderophore elaborated by others. The soybean symbiont *Bradyrhizobium japonicum* 6IAt52 was reported to utilize a large number siderophores produced by other microorganisms to scavenge iron, facilitating its survival in the rhizosphere (Plessner *et al.,* 1993).

In order to utilize a particular siderophore, specific uptake system is needed to transport the ferri-siderophore complex. The complex is transported across the microbial membrane through a high-affinity outer membrane-bound receptor. In gram-negative bacteria these receptors are FepA in *Escherichia coli* and ViuA in *Vibrio cholera.* Transport of the ferri-siderophore into cell is dependent upon TonB along with specific periplasmic (FepB) and inner membmne proteins (Klebba *et al.*, 1993, Sprencel *et al.*,2000). In *Sinorhizobium meliloti* a gene cluster termed rhtA was identified which encoded the outer membrane receptor for the siderophore rhizobactin 1021 (Lynch *et. al.*,2001).

Many rhizobial strains appear to have no high-affrnity iron acquisition system and utilize polymeric form of iron via low affinity iron transport system without involvement of any solubilizing and transporting compounds. As such, it is tempting to conceive that introduction of such organisms with genes required for high-affinity iron acquisition would make them more efficient in utilization of insoluble iron. *Rhizobia* with a high-affinity iron acquisition system are suggested to be more efficient in N_2-fixation bansforming the host to be high yielding in N_2 deficient soils. Earlier Gill *et al.,* (1991) reported that *Sinorhizobium meliloti* mutants unable to produce or transport siderophores were impaired in nitrogen fixation relative to wild type when plants were examined after 70 days.

Rhizobium strain BICC 651, which was isolated from a nodule produced on the root of chickpea (*Cicer arietinum* L.) plant produced a catechol siderophore and its cognate membrane-bound receptor in response to iron deficiency (Roy *et al.,* 1994).In the present study two siderophore over-producing mutants of the strain were isolated by selection on chrome azurol sulfone (CAS) plate (Schwyn and Neiland, 1987) following fiansposon mutagenesis. Supression of siderophore production of these mutants was compared to that of the parent strain in liquid medium supplemented with increasing concentrations of iron to identify the nature of the mutants. One of these mutants was chanacterized for its uptake of ^{59}Fe-siderophore complex within the cell and compared with that of the parent strain. The Tn5 adjacent genomic DNA region of the mutant was identified. The mutant was also examined for nodulation to determine the relative importance of siderophore utilization during symbiosis.

MATERIALS AND METHODS

Bacterial strains, plasmids and media– Bacterial strains and plasmids used in the study are listed in Table 1. Complete medium described by Modi et al- (1985) [composition (g/l): K_2HPO_4. 0.5; $MgSO_4$. $7H_2O$,0.4; NaCl, 0.1; mannitol, I0; glutamine, I; and NH_4+NO_3, I; pH 6.81 was used to study the siderophore production of the strain BICC 651 and its mutants. The medium was deferrated with hydroxyquinoline (Rosenberg, 1979). To prepare I litre of blue CAS-agar (Schwyn and Neiland, I987), 60.5 mg of CAS was dissolved in 50 ml of water and mixed with I0 ml of ferric iron solution (I mM $FeCl_3$. $6H_2O$ in 10 mM HCl). Under stirring this solution was slowly added to72.9 mg of hexadecyl trimethyl ammonium bromide (IDTMA) dissolved in 40 ml of water. The resultant dark blue liquid was autoclaved. Separately 900 ml of Complete medium was prepared to which 30.24 gof pipes was added. The pH of the medium was adjusted to 6.8 with 10 N NaOH. To this medium 20 g of agar was added and then autoclaved. After cooling to 50°C, the dye solution was added along the glass wall, with enough agitation to achieve mixing without generation of foam. Each plate received 25 to 30 ml of blue agar.

Isolation of transposon insertion mutants

Escherichia coli S 17.1, containing the suicide plasmid pSUP5011 which carries the transposon Tn5 with broad host range mobilization sequence (mob), was used as donor in conjugative cross. The *E. coli* strain was resistant to the antibiotic, neomycin due to the presence of Tn5 and the recipient *Rhizobium* BICC 651, was sensitive to the antibiotic. As such neomycin (50 μg/ml) was used as the marker for the donor selection. In contras! the *E. coli* strain was highly sensitive to rifampicin. To use rifampicin (100 μg/ml) for selection of recipient during transconjugation, a rifampicin resistant spontane-

ous mutant of the strain BICC 651 was selected and designated as *Rhizobium* BICC 651R. The donor, *E. coli* S 17.1, and the recipient *Rhizobium* BICC 65 I R, were grown separately in Luria broth (LB) and yeast extract mannitol (YEM) broth respectively to their exponential phase, mixed, centrifuged to collect the cells and spread onto plates containing tryptone-yeast extract agar medium [composition (g/l): bactotyptone, 8; bacto yeast 5; NaCl, 5; and agar 20; pH 6.8] (Beringer, 1974). The mating mixture was incubated overnight at 30°C. The cells were then collected and suspended in YEM broth and plated on *Rhizobium* medium (Himedia, India) containing neomycin (50 μg/ml) and rifampicin (100 μg/ml). The plates were incubated at 28°C for 3 to 4 days. Thereafter, the colonies of the transconjugants were transferred to a master plate.

Screening of siderophore over-producing (Sid^{++}) mutants

From the master plate, the transconjugants were transferred to CAS plate by replica plating and incubated at 28°C for 7 to 14 days for development of an orange halo around the colonies. The Sid^{++} mutants were identified by comparing the diameter of orange halo produced by the mutants with that of the parent strain BICC 651R.

Effect af increasing concentrations af $FeCl_3$ on growth and siderophore production

Sid* mutants were grown in deferrated Complete medium in presence of increasing concentrations of $FeCl_3$ (0-100 FM). Following 48 hours of incubation at 28°C on a shaker, growth of the mutants was estimated by measuring the OD_{590} of the cultures and siderophore production was assayed using the CAS reagent (Schwyn and Neiland, 1987).

Assay of ferri-siderophore complex uptake

The uptake buffer [50 mM potassium phosphate, pH 7.0; A.2 mM nitrilotrimetate (NTA); 0.025 yo disodium succinate hexahydratel was made iron- limited by extraction with hydrokyquinoline. One nmole of $^{59}FeCl_3$ in 2.5 μl volume (specific activity 4.22 Ci/g)was mixed with 21 nmole (15 μl) of siderophore. The molar concentration of siderophore was measured based on concentnation of deferoxamine mesylate (Sigma Chemical Company, USA) using the CAS reagent. Following incubation for I0 min at room temperatue the volume of the mixture was made to 100 pl with uptake buffer and the mixture was kept for an hour under shaking at room temperature. The parent stain, BICC 651R and one of the putative uptake mutants, B001, were grown in deferreted Complete medium to the late exponential phase. The cells were harvested, washed twice with the uptake buffer and suspended in the same buffer to an OD_{590} of 0.5. To 10 ml of the cell suspension 100 μl of $^{59}FeCl_3$ sideophore complex containing one nmole of ^{59}Fe was added. The assay mixture was incubated at 32°C on a shaker water bath. One ml aliquot of the assay mixture was withdrawn at regular interval, quickly added to I0 ml of prechilled wash buffer [50 mM potassium phosphate, pH 6.8; 0.4 mM NTA], and filtered through membrane filter (0.45 pm; Millipore, USA) presoaked in uptake buffer for at least one hour before the experiment. The filter was washed with another I0 ml of the wash buffer, dried and then counted in a liquid scintillation system (Beckman LS 5000 CE) using cocktail 'O' (Spectrochem, India) scintillation fluid.

DNA manipulations

Bacterial genomic DNA preparation, restriction digestion and agarose gel electrophoresis were carrid out using standard protocol (Sambrook *et al.*, 1989). To estimate *Bam* HI restricted Tn5 adjacent genomic DNA fragment in the mutant B001, Southem hybridization was performed using a biotin labeled probe prepared from inverted repeats (IR) border region of Tn5 fragment following the manufacturer's instruction (NE Blot Phototope Kit Biolabs). The DNA band showing positive signal was eluted from the gel using DNA purification kit (Genei, India). The digested DNA fragments were allowed to self-ligate at a concentration of 0.3-0.5 μg/ml in presence of 3 U/ml of T_4 DNA ligase (Promega) overnight at 4°C (Hvang *et al.*, 2000). The ligation mixture was extracted with phenol: chloroform, precipitated with ethanol, and resuspended in sterile distilled water to a concentration of 20 pdml. PCR primer pair from the IR : border region of Tn5 sequence (Genbank accession no. U00004 L19385) in outward direction,

Tn5Int (CGGGAAAGGTTCCGTTCAGGACGC) and downstrsm region of *Bam*HI site of Tn5, Tn5BR (CATTCCTGTAGCGGATGGAGATC) was designed. The inverse PCR was carried out using the self-ligated product containing a portion of the Tn5

as template and Tn5Int and Tn5BR as primers. The amplifications were performed using Perkin-Elmer PCR system 2400 in 50 µl reaction mixture containing I X enzyme buffer, 1.5 mM $MgCl_2$,200 µM dNTPs (each), 500 nM primers (each) and 1.5 U Taq polymerase (Fermentas, USA) and 50 ng of purified self-ligated product. The thermal prognamme used for PCR was as follows: initial denaturation for 10 min at 94°C;35 cycles of denaturation for 30 sec at 94°C, annealing for 30 sec at 55°C, and extension at 72°C for 1 min, followed by a final extension at 72°C for 10 min. The identity of the PCR product was confirmed by nested PCR and also by ascertaining the profile of bands resulting from its specific endonuclease digestion. The PCR product was purified and sequenced by ABI PRISM 377 automated DNA sequencer (Perkin-Elmer, Applied Biosystem, Inc.)- Tn5 adjacent genomic DNA sequence was analyzed using BLAST version 2.2.1 of National Center for Biotehnolory Information. Nucleotide BLAST and BLASTX were used to search for nucleotidè sequences and derivative amino acid sequences, respectively.

Plant inoculaion and nodulation assay For nodulation to occur surface-sterilized seeds of chickpea were inoculated with *Rhizobium* strains and planted in sea sand in pots. Sea sand was treated with HCl, washed with distilled water until the pH of the washing was neutral. It was then air dried and fumigated with chloroform overnight under airtight condition, autoclaved and finally incubated at 2@t in a hot air oven for six hours. Five hundred gram of sterile sand in an earthen pot was moistened with 100 ml of nitrogen free plant nutrient medium for planting of the seeds. The composition of nitrogen fue medium was (g/l): $CaSO_4$. $2H_2O$,0.34; K_2HPO_4. 0.17; $MgSO_4$. $7H_2O$,0.25; Fe Citrate, 0.002; KCl, 0.075; trace element solution, 0.5 ml; pH 7.2 (Norris and Date, 1976). The composition of the trace element solution was (g/l): $ZnSO_4$. $7H_2O$, 2.25; $CuSO_4$. $5H_2O$, 1.00; $MnSO_4$. $5H_2O$, 0.50; $CaCl_2$. $2H_2O$, 2.00 $Na_2B_4O_7$. $10H_2O$, 0.23;

$(NH_4)_6Mo_7O_{24}$, 0.10. Surface sterilization of chickpea seeds was carried out by soaking in concentrated H_2SO_4 for l0 minutes, washed several times with sterile distilled water. The parent strain BICC 65IR and its Sid*" mutant B00l were grown in YEM broth till late-log phase, and the harvested cells were mixed with sand+charcoal (1:3) containing 2 % aqueous sodium carboxymethyl cellulose. The mixture was used to coat the surface-sterilized seeds. The coated seeds (~10^8 bacteria/ seed) were kept in dark for overnight and the next day five seeds were transferred to each pot containing the sterile sand soaked in nitrogen free plant nutrient medium. A control set that did not receive any inoculum was also included in this study. The pots were kept in well-illuminated condition and watered when necessary to moisten the sand. At one week interval, 50 ml of 1/10^{th} dilution of nitrogen free plant nutrient medium was added to each pot. At 35 days of inoculation, plants were uprooted and observed for development of nodules.

RESULTS

Tn5 mutagenesis, and kolation of Sid^{++} mutants

Random mutagenesis of the *Rhizobium* strain BICC 65IR by mobilization of Tn5-mob from the suicide vector pSUP50l I to the recipient cells occurred at a frequency of $l0^{-5}$ per donor as revealed by the number of neomycin-resistant transconjugants. This is about 1000 times geater than that of spontaneous resistance of the recipient to neomycin (10^{-8}). Upon screening of 1,000 transconjugants from two independent mating experiments, two siderophore ing mutants were isolated. The mutants produced larger orange haloes on CAS-agar plate as compared to that of their parent (Figure 1).

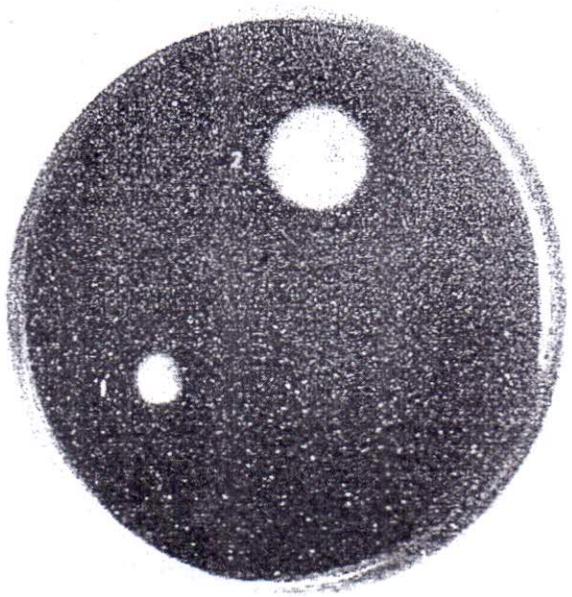

Fig. 1 : Siderophore production on CAS plate by *Rhizobium* BICC 65IR (l) and its siderophore over-producing mutant B001 (2).

Table 1 : Bacterial strains and plasmids used in the study

Strains and plasmids*	Relevant characteristic(s)[a]	Reference and/or source
Escherichia coli SI7.I	Conjugative donor for pSUP5011	Laboratory collection.
Rhizobium BICC 651	Wild type, Sid^{++}	Roy *et. al.* 1994.
Rhizobium BICC 65IR	Sid^{+} :: Rif^{3} (spontaneous)	This study.
Rhizobium B001	Sid^{++} :: Tn5-mob, Rif^{3} Neo^{r}	This study.
Rhizobium B003	Sid* Tn5-mob, Rif^{3} Neo^{r}	This study.
*Plasmid pSUP5011	pBR325(Bam·)::Tn5-mob, Ap^{r} Neo^{r} Cm^{r}	Simon, 1984.

[a]Ability to produce siderophore is denoted by Sid^{+}, siderophore over-production is denoted by Sid^{++}; resistance to antibiobiotics such as rifampicin, neomycin, ampicillin and chloramphenicol are denoted by Rif^{3}, Neo^{r}, Ap^{r} and Cm^{r}, respectively.

Effect of increasing concentrations of $FeCl_3$ on the growth and siderophore production

To estimate siderophore production of the Sid^{++} mutants in the presence of iron, they were grown in deferrated Complete medium supplemented with increasing concentrations of $FeCl_3$ (0-100 μM).

Growth and siderophore production of the strains were ascertained at 48 hours of incubation and compared with those of the parent strain BICC 65IR

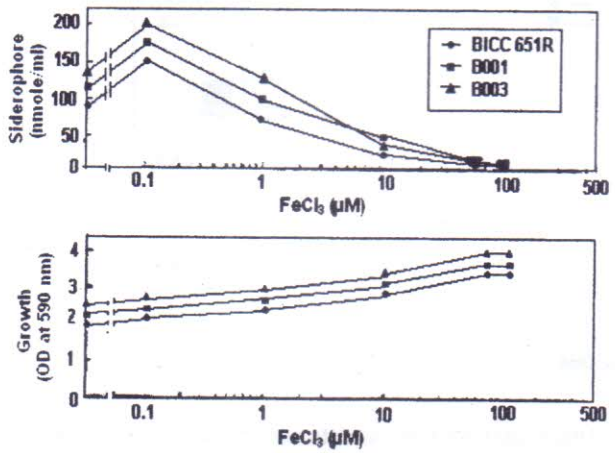

Fig. 2 : Effect of increasing concentrations of $FeCl_3$ on gowth and siderophore production of *Rhizobium* BICC 651R and its Sid^{++} mutants (B001 and B003) in the deferrated Complete medium.

(Figure 2). In deferrated medium without iron supplement the biomass yields of the parent shain BICC 65IR and its Sid* mutants (strains B00l and 8003) were low as compared to those in the iron-supplemented medium. The bacterial growth improved when the medium was supplemented with increasing concentrations of $FeCl_3$ up to 50 μM. In deferrated medium supplemented with 100 μM $FeCl_3$, there was no discernible difference in the biomass yield of the strains as compared to that in the medium with 50 μM $FeCl_3$. The level of siderophore as measured by CAS assay in the medium without iron supplement was 76, 104 and 124 nmole per ml for the parent strain BICC 65IR and the mutants, B001 and B003, respectively after 48 hrs (Figure 2). In the medium with 0.1 μM $FeCl_3$, however, maximum production of

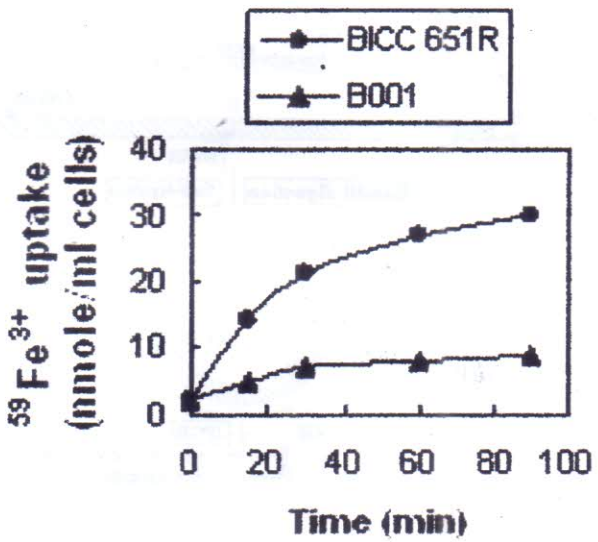

Fig. 3 : Uptake of $^{59}Fe^{3+}$ siderophore complex by *Rhizobium* BICC 65IR and its putative uptake mutant, B001.

siderophore was observed and the level was 140, 180 and 200 nmole per ml for the strain BICC 65IR and the mutants, B001 and B003, respectively. With increasing concenffations of iron the level of siderophore decreased gradually and at 50 μM $FeCl_3$ the siderophore was almost undetectable in the culture filtates. The pattern of supression of siderophore production with increasing concentrations of $FeCl_3$ in the two Sid^{++} mutants was very similar to that of the parent strain BICC65IR. These results suggested that the mutants could not be

Table 2 : Symbiotic performance of *Rhizobium* BICC 651R and its siderophore over-producing mutant

Strains	No. of nodules per plant	Wet weight of nodules (mg) per plant	Root length (mm)	Shoot length (mm)
BICC 651R	20	872.5	100	230
8001 (sid^{++})	26	2072.9	130	230
Control	Nil	Nil	80	200

Measurements were made on the 35th day of sowing

considered as regulatory ones, as such were putatively considered to be uptake mutants.

Siderophore mediated ion uptake

Uptake of ferri-siderophore complex by one of the putative uptake mutants, 8001, was studied to assay its iron uptake ability mediated by the siderophore. The mutant was incubated in the presence of ^{59}Fe-siderophore complex and the radioactive iron taken up by the cells was measured. As shown in figure 3 the rate of uptake of radioactive iron by the mutant is much slower and the highest level of iron uptake by the mutant (10 nmole/ml cells) is one-third as compared to that of the parent strain (30 nmole/ml cells).

Identificaion of the gene in Sid^{++} mutant

A 3.4 kb *Bam*HI restricted genomic DNA fragrnent of the Sid^{++} mutan! B001, containing the 2.7 kb Tn5 posterior fragment (without Neo'gene), was found to hybridize with the probe prepared from the IR border region of Tn5 fragment. The DNA band was eluted from the gel and allowed to self-ligate. The Tn5 adjoining genomic DNA was amplified by inverse PCR using the Tn5Int and Tn5 BR primers. An 834 bp amplicon was obtained which contained 717 bp genomic DNA with Tn5 sequence at its upstream as well as at its downstream negions (Figure 4). The amplicon was sequenced using the Tn5 BR primer and the sequence of the genomic DNA region is shown in figure 5. Using nucleotide BLAST, &e 717 bp genomic DNA sequence showed highest similarities (78 %) with nucleotide sequence of linear chromosome of *Agrobacterium* sp. H-13 (GenBank accession no. CPAA2249). Upon BLASTX search, the derivative amino acid sequence of the Tn5 adjacent 265 bp out of the 717 bp genomic DNA was found to match with the end portion (193 to 279) af amino acid sequence of siderophore interacting protein of *A. tumefaciens*

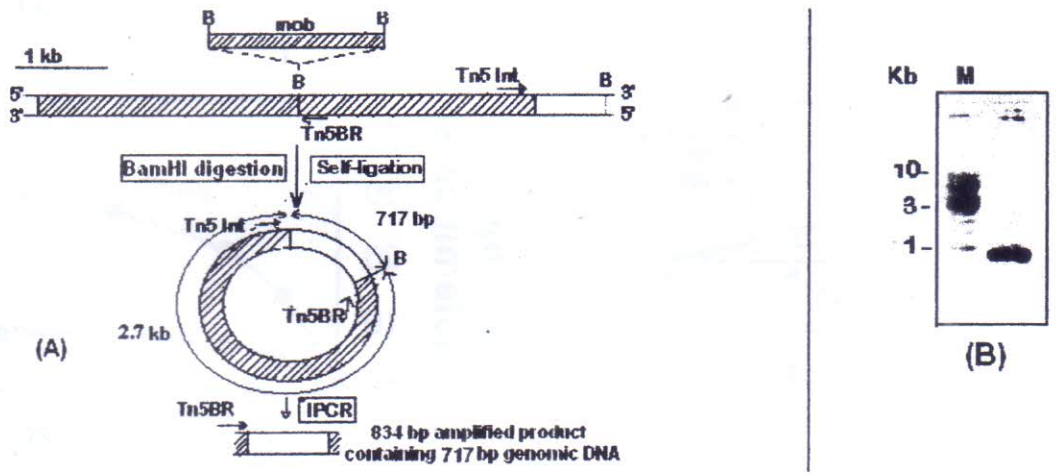

Fig. 4 : (A) Strategy of inverse PCR of Tn5 containing *Bam*HI fragment of the mutant B001. B, *Bam*HI; dashed area, Tn5 fragment; white area genomic DNA; Tn5BR and Tn5Int primers were used for PCR and sequencing; (B) Ethidium bromide stained agarose gel showing 834 bp PCR product of B001.

CCNWGSO2S6 [Identities= 90%; Frame=+2; Length =279; GenBank accession no. EHHO7I I7] (Figures 6 and 7). Alignment of the codons of the deduced amino acid sequence with the nucleotide sequence worked out predicted the ORF disrupted by Tn5 to end at nucleotide 263. The 265 bp gene fragment of the *Rhizobium* strain BICC 65IR was named as siderophore interacting protein (*iup 1*) and submitted to GenBank under the accession no. GU549413.

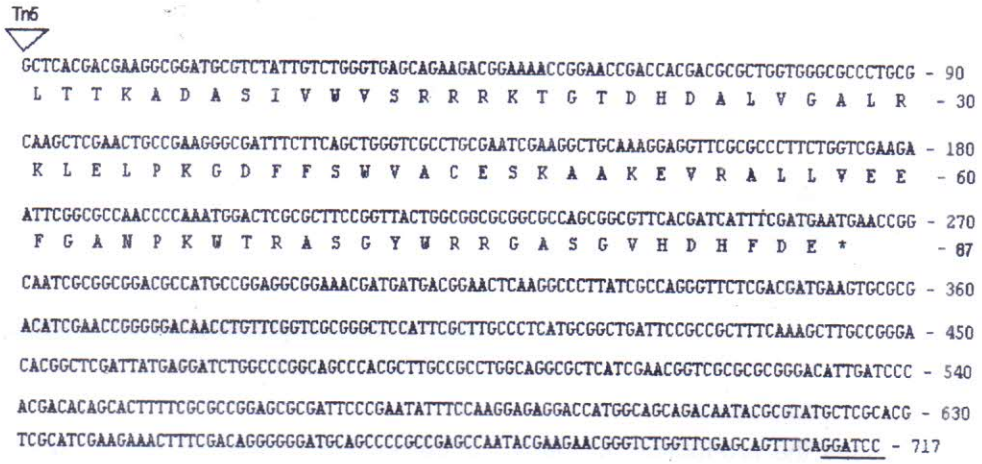

Fig. 5 : Nucleotide sequence of 717 bp genomic DNA containing 265 bp of posterior end of an ORF (homologous to gene of siderophore utilization protein) adjacent to Tn5 in the strain B001 (Sid^{++} mutant). The deduced amino acid sequence of the ORF fragment is also shown. *Bam*HI restriction site present at the posterior end of 717 bp genomic DNA fragment is underlined.

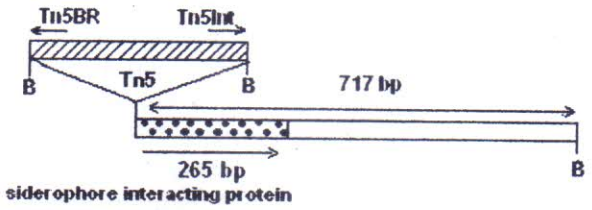

Fig. 6 : Physical map of Tn5 adjoinng *Bam*Hi. fragment of the strain B001 showing transcriptional direction of gene codes for siderophore interaction protein. B, *Bam*HI; dashed box, Tn5 fragment; dotted box, genomic DNA with posterior end of gene encodes for siderophore interacting protein; white box, other portion of genomic DNA not identified in the study; Tn5BR and Tn5Int primers were used for PCR.

Plant nodulaion study

Table 2 presents the data on symbiotic performance of the siderophore over-producing mutant B001 and its parent strain BICC 651R. The control plants receiving no inoculum produced no nodules and the length of both of the roots and the shoot was shorter as compared to those of the inoculated plants. The average number of nodules produced per plant by the mutant B001 was almost 30% higher than those produced by the parent strain BICC 651R. The nodules produced by the mutant B00I as compared to those of its parent were larger and considerably heavier.

DISCUSSION

Part of life cycle of *Rhizobium* requires invasion, growth and differentiation within plant tissue and the role of high affinity iron transport may be important in this plant microbe interaction (Gill, Jr. *et al.* 1991). The high affinity iron transport system consists of two basic components. The synthesis and secretion of a low molecular weight ferric-specific chelation agent to solubilize iron, termed siderophore and a membrane transport rystem that recognizes the ferric complex of the particular siderophore (Roy *et al.*, 1994). The production of the system is induced and is required for growth under iron limiting condition.

Siderophore over-producing mutants could be either of the two types: (i) mutants having Tn5 insertion in their siderophore regulatory genes, thereby constitutivety producing the siderophore without iron regulation and causing an increased level of siderophore in the medium, and (ii) mutants affected in their transport components causing a failure in the internalization of ferri- siderophore complex resulting in an increased level of siderophore in the medium. To characterize this type of the Sid^{++} mutants the strains were grown in increasing concentrations of $FeCl_3$. It is well known that iron starvation induces the production of siderophores and

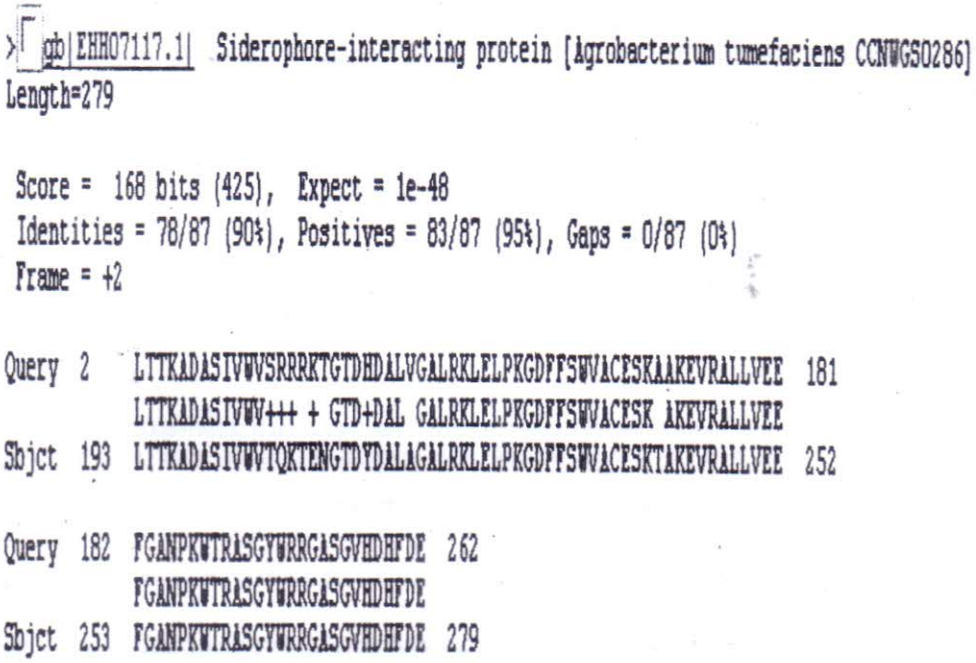

Fig. 7 : Alignment of deduced amino acid sequence of Tn5 adjacent genomic DNA of B001 (Query) with that of siderophore interacting protein of *Agrobacterium tumefaciens* (Subject).

iron repletion suppresses the production. In many systems as well as in rhizobia gene products (enzymes) responsible for siderophore production are optimally expressed when iron is provided only at a low concentration. In the present study siderophore production of the strain BICC 65IR was optimum at a concentration of 0.1 μM of $FeCl_3$ and then it decreased with increasing concentrations of $FeCl_3$ and at a concentration of 50 μM siderophore production was minimal (Figure 2). The two Sid^{++} mutants, B001 and B003, also showed a pattern of siderophore production similar to the parent strain, as such were not considered to be regulatory mutants. Although siderophore production, decreased, growth of the strains increased with increasing concentrations of $FeCl_3$ supplemented to the deferrated Complete medium (Figure 2) indicating the iron requirement of the strains.

One representative Sid^{++} mutant B001, putatively identified as not being a regulatory one, was examined for its ferri-siderophore uptake and was found to be impailed in its iron uptake capacity. The highest level of iron uptake by the mutant was about one third of that of the parent strain. The Sid* phenotype of the mutant was due to failure of effectivety internalizing iron bound form of the siderophore to within the cell, as such siderophore accumulates in the medium (Figure 3). The reduced ability of the Sid^{++} mutant to transport ferri-siderophore complex explains the increased level of siderophore in the medium. In the Sid^{++} mutant, B001, Tn5 disrupted the genomic region which codes for a siderophore interacting protein (Figure 6) and resulted in an impaired uptake of ferri-siderophore complex. It was observed that out of 717 bp genomic DNA, derivative amino acid sequences of only 265 bp adjacent to Tn5 showed W yo similarity with siderophore interacting protein of A. tumefaciens CCNWGSO2S6 (Figures 6 and 7). The function of the rest of the genomic fragment is as yet unknown to us.

The *Rhizobium* strain BICC 651 is a fast growing one and was isolated form a root nodule of chickpea plant. The bacterium profusely nodulates its host which may be due to a selective advantage conferred by its siderophore production in the rhizosphere. Its siderophore over- producing mutant thus, may be better adapted for iron sequestration in the microenvironment of host rhizosphere and rhizoplane which may be intimately related to the production of nod factors resulting in production of higher number of nodules than its parent (Table 2). These results is in congruent with those of Raychaudhuri *et al.*, (2005) who also reported a siderophore over- producing mutant strain NI5 gen-

erated by N-methyl-N'-nitro-N-nitrosoguanidine mutagenesis of *Mesorhizobium ciceri* was symbiotically more efficient than its parent. Barton *et al.* (1992) found a correlation between dinitrogen fixation and rhizobactin producing capability of *Sinorhizobium meliloti* strain 1021. However, Manjanatha *et al.,* (1992) observed that Tn5-induced siderophore over-producing mutants of *Sinorhizobium fredii* were less competitive than their parent strain.

Benson *et al.,* (2005) reported that the gene *feg*A, required for utilization of ferrichrome when transferred from *Bradyrhizohium japonicum* 61A152 into *Mesorhizobium* sp. GN25 and *Rhizobium* sp. STI, the transconjugants developed the ability to utilize ferrichrome, thus competed for iron with indigenous soil bacteria and increased their stability in rhizospheric soil. Subsequently, enhanced nodule occupancy as well as high yield of the legumes was observed when the crops lvere inoculated with such hansconjugants. Having worked out the nucleotide sequence of an iron uptake gene in the strain BICC 65IR it should be possible to mutate the genes at will by site directed mutagenesis. It should also be possible to copy tfte gene and transfer the copies to strains lacking it and the rccombinants would possibly be able to uptake siderophores produced by other microorganisms for their own iron rquirement and be potentially more competent in their symbiotic efficiency.

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