Polish Journal of Microbiology
2005, Vol. 54, No 1, 37–41

Symbiotic Effectiveness of a Siderophore Overproducing Mutant of *Mesorhizobium ciceri*

NUPUR RAYCHAUDHURI[1], SUBRATA K. DAS[2]
and PRAN K. CHAKRABARTTY*

Department of Microbiology, Bose Institute, P1/12, CIT Scheme VII M,
Kolkata 700 054, India

Received 29 July 2004, received in revised form 22 November 2004,
accepted 3 December 2004

Abstract

Mutants of *Mesorhizobium ciceri* BICC 651 were generated by *N*-methyl-*N*'-nitro-*N*-nitrosoguanidine mutagenesis. Siderophore overproducing mutants were identified on Chrome azurol S agar plates. One of them determined as N15 was examined for symbiotic efficiency and compared to its wild type parent *i.e.* BICC 651 strain under sterile conditions using Leonard jars in growth chamber and also in pots containing nonsterile alluvial field soil. It was observed that the strain N15 produced about 30% higher number of nodules per plant, fixed 25% more nitrogen per gram of nodule and caused more than 30% increased dry weight of plant shoots.

Key words: *Mesorhizobium ciceri*; siderophore overproducing mutant; nodulation; nitrogen fixation

Introduction

In most aerated soil at neutral or alkaline pH, solubility of inorganic iron is too low to support good growth of microbes and plants. Because under these conditions iron is present in the unavailable oxidized form [Fe^{3+} or the $Fe(OH)_3$] many microorganisms developed a strategy to solubilize and uptake the unavailable iron by producing low molecular weight (500–1000 Da) ferric iron complexing agents, the siderophores (Neilands, 1981). The siderophores are produced in response to iron starvation and form complexes with Fe^{3+} iron. These complexes are taken by the cells to meet their iron requirement.

Leguminous plants undergo a symbiotic relationship with respective root nodule bacteria. This relationship is iron dependent, since iron is required for nodule formation as well as synthesis of components, such as nitrogenase complex and leghaemoglobin, required for nitrogen fixation. Number of nodules decreased and nodule initials remained depressed under low iron condition (Tang *et al*., 1990). In peanuts iron deficiency arrested nodule development and also decreased nitrogenase activity in already developed nodules (O'Hara *et al*., 1988a). Effective nodulation has been shown to depend on the ability of the infecting rhizobium to obtain iron from soil. Strains of rhizobium vary in their ability to acquire iron for nodule initiation and development (O'Hara *et al*., 1988b). It has been suggested that rhizobia, which produce siderophore to aid in iron chelation and uptake, may be more competitive in environment poor in iron. Following nodulation, however, the iron requirement of the bacteroids is met from the host legume.

In this study, a siderophore overproducing mutant, N15, obtained by chemical mutagenesis was evaluated and compared with its wild type parent, *Mesorhizobium ciceri* BICC 651, as to their symbiotic activities. *M. iceri* BICC 651 produces extracellularly a catechol-type siderophore containing 2,3-dihydroxybenzoic acid as the core compound in iron-deficient medium (Roy *et al*., 1994).

[1] Present address: Department of Botany, Visva Bharati, Santiniketan 731 235
[2] Present address: Institute of Life Sciences, Bhubaneswar, Orissa 751 023
* Corresponding author: Tel: 91-033-2337 9416, Fax: 91-033-2334 3886, E-mail: krishna@boseinst.ernet.in

Experimental

Materials and Methods

Media and growth conditions. Bacterial strain, *M. ciceri* BICC 651, was obtained from our laboratory collection (Roy *et al.*, 1994). Siderophore overproducing mutant N15, was generated by *N*-methyl-*N*'-nitro-*N*-nitrosoguanidine (NTG, 50 mg ml^{-1}) mutagenesis of the wild type *M. ciceri* strain BICC 651. The bacterial strains were grown aerobically in complete medium (Modi *et al*., 1985) with the following composition (per liter): K_2HPO_4, 0.5 g; $MgSO_4$ X $7H_2O$, 0.4 g; NaCl, 0.1 g; mannitol, 10 g; glutamine, 1 g and NH_4NO_3, 1 g. The medium was deferrated by treatment with 8-hydroxyquinoline following the method of Rosenberg (1979). During inoculation the initial cell number of the cultures was adjusted to 10^7cells ml^{-1}. Bacteria were subcultured in deferrated complete medium to reduce their internal ferric iron stores before use as inoculum.

The CAS Blue Agar medium was prepared following the method of Schwyn and Neilands (1987) replacing MM9 growth medium with complete medium. In the culture filtrate catechol type of siderophore produced by the strains was measured quantitatively following the method of Arnow (1937) using 2,3-dihydroxy benzoic acid (DHBA) as the standard.

Isolation of mutants. The strain BICC 651 was grown in complete medium without deferration to mid-log phase, when the culture reached OD_{590} from 0.7 to 0.8 (4.8×10^8 cells). The cells were harvested by centrifugation at 10,000 g for 10 min at 4°C, washed twice with 0.1 M sodium citrate buffer, pH 5.5, and resuspended in fresh sodium citrate buffer. To the cell suspension NTG was added to a final concentration of 50 μg ml^{-1} and the suspension was incubated at 28°C. Aliquots were taken at intervals, centrifuged and washed twice with 0.1 M phosphate buffer, pH 7.0. The washed cells at appropriate dilution were plated on complete medium solidified with agar for determination of survival of cells percent.

At 50% survival cells were aseptically resuspended in complete medium without deferration and incubated for 12 h for segregation. The cells were then collected, plated on CAS-agar and incubated for two days. Siderophore overproducing mutants, eliciting larger orange halo zones around their colonies than the wild type parent, were isolated after visual inspection.

Plant infection test. Seeds of chick pea (*Cicer arietinum*) cv. Pusa C-235 were surface-sterilized with 0.1% $HgCl_2$ for 3 min, washed several times with sterile distilled water, treated for 3 min with 75% ethanol and rinsed in sterile water. The wild type parent, *M. ciceri* BICC 651, and its siderophore overproducing mutant N15, were grown for 24 h and the harvested cells were used for plant infection.

Before planting in soil the seeds were soaked in water for 1 h and then coated with bacteria mixed with soil-charcoal (1:3) containing 2% aqueous sodium carboxymethyl cellulose. The coated seeds (10^6 bacteria/seed) were kept in dark for overnight and the next day five seeds were sown in each pot of field soil. The soil was moistened with distilled water, plants were uprooted after 35 days for counting the number of nodules and the growth parameters of the plants. The coated seeds were also planted aseptically in sterile vermiculite-quartz sand mixture (1:1) using Leonard jar assemblies (Leonard, 1943). Each jar with three plants received 400 ml of nitrogen free nutrient solution as described previously (McKnight, 1949). Plants were grown in a controlled growth chamber with 12 h light and 12 h dark. Day lighting was 250 microeinsteins/$m^2\times sec^{-1}$. Day temperature was 23°C, night temperature was 15°C and the relative humidity was 60%. After 35 days shoot portions of the plants were cut at the root-stem juncture and placed in an oven at 70°C for five days and then weighed for estimation of shoot dry weight.

Roots with attached nodules were placed in 50 ml culture tubes. The tubes were capped with suba seal. Air (5.0 ml), was removed from each tube to which 5.0 ml of acetylene was then injected. Gas sample (1.0 ml) was withdrawn at 1 h of incubation and ethylene produced was determined using a Nucon 5700 gas chromatograph equipped with a 1.6×6 mm Porapak R column and a flame ionization detector at 100°C at a nitrogen carrier gas flow of 35 ml per min.

Results and Discussion

Growth and siderophore production. Siderophore overproducing mutants of BICC 651 strain were isolated by treatment with NTG. Fifty percent of the cells survived 15 min contact with NTG (50 μg ml^{-1}). The surviving cells were allowed to segregate and then screened for siderophore overproduction on CAS agar plates. Figure 1 shows that siderophore production by the overproducing mutant N15 (colony 2) is much higher than that of the wild type (colony 1) as revealed by orange halo zones surrounding the colonies.

During stationary phase of growth, in the deferrated complete medium, OD_{590} of BICC 651 strain was higher than 2 (Fig. 2A), whereas of N15 strain it was almost 4 (Fig. 2B). The level of siderophore in the BICC 651 culture was 110 nmol equivalent of DHBA ml^{-1} (Fig. 2A) as compared to 400 nmol ml^{-1} for N15 (Fig. 2B) was is in agreement with the results obtained with CAS agar plate.

Effect of iron on growth and siderophore production. The wild type and the siderophore overproducing mutant were grown in the deferrated complete medium with increasing concentrations of iron. In the deferrated medium without iron supplement the biomass yields of the wild type strain BICC 651 and its siderophore overproducing mutant N15 were low as compared to those in the iron-supplemented medium. The biomass yields of both strains increased with increasing concentrations of iron up to 50 mM $FeCl_3$ (Fig. 3). When deferrated medium was supplemented with 100 mM $FeCl_3$ there was no discernible difference in the growth of the strains. Siderophore productions in the medium without iron supplement were after 36 h 90 and 410 nmol equivalent of 2,3-DHBA ml^{-1} for the wild type and the mutant N15,

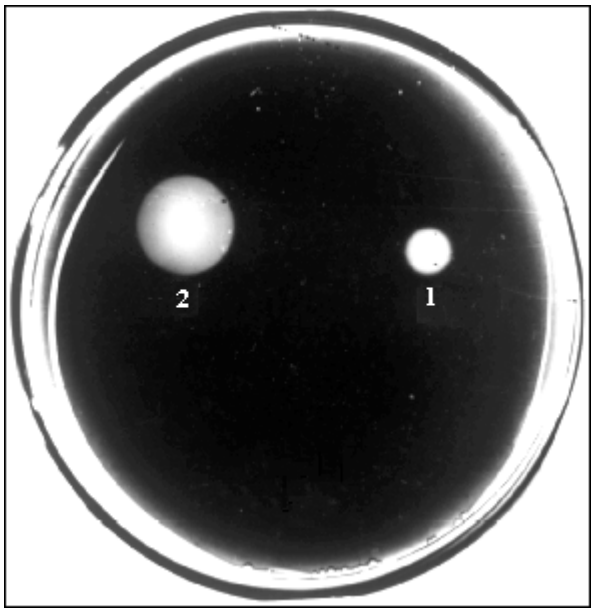

Fig. 1. Assessment of siderophore production on CAS agar plate by the wild type *Mesorhizobium ciceri* BICC 651 (Colony 1) and the mutant N15 (Colony 2).

respectively (Fig. 3). In the medium with 0.1 mM $FeCl_3$, however, maximum production of siderophore were observed and were 145 and 455 nmol equivalent of DHBA ml^{-1} for BICC 651 and N15, respectively (Fig. 3). With increasing concentrations of iron the level of siderophore produced decreased gradually and at 100 mM $FeCl_3$ the siderophore was almost undetectable in the culture filtrates. The pattern of repression of siderophore production with increasing concentrations of $FeCl_3$ was very similar in both strains (Fig. 3). This suggests that the overproducing mutant is defective in the uptake of ferric complex (Gill and Neilands, 1989).

Effect of plant inoculation on nodulation and nitrogen fixation. Table I shows the effect of inoculation of *Cicer arietinum* plants with *M. ciceri* BICC 651 and its siderophore overproducing mutant N15, under sterile, controlled conditions in a growth chamber. As expected, control plants receiving no inoculum produced no nodules and the average dry weight of shoots was very low as compared to inoculated plants. The average number of nodules produced per plant by N15 strain of *M. ciceri* was almost 30% higher than by the wild type BICC 651. The nodules with N15 strain reduced almost 25% more acetylene than those produced by BICC 651 strain per unit weight (Table I).

In the nonsterile soil no discernible difference was observed between uninoculated plants receiving no bacterial inoculum and plants inoculated with the strain BICC 651 in respect of shoot length, shoot weight, nodule number or nodule weight (Table II). However, plants inoculated with N15 strain consistently produced almost 35% more nodules of larger biomass and 25% or more increased shoot weight than plants inoculated with BICC 651 (Table II). The nodules produced by the overproducing mutant N15 as compared to the wild type were bigger and considerably different in the total weight of the nodules produced per plant. The average root length of the hosts inoculated with the siderophore overproducing mutant was smaller but their weight was higher than those of plants treated with the wild type (Table II).

M. ciceri strain BICC 651 profusely nodulates its host which may be due to a selective advantage conferred by its siderophore production in the rhizosphere. The role of bacterial siderophores on a nodulation and nitrogen fixation process was examined in the present study. It is evident that the siderophore overproducing mutant of *M. ciceri* is more efficient in a nodule formation on its host than its wild type parent BICC

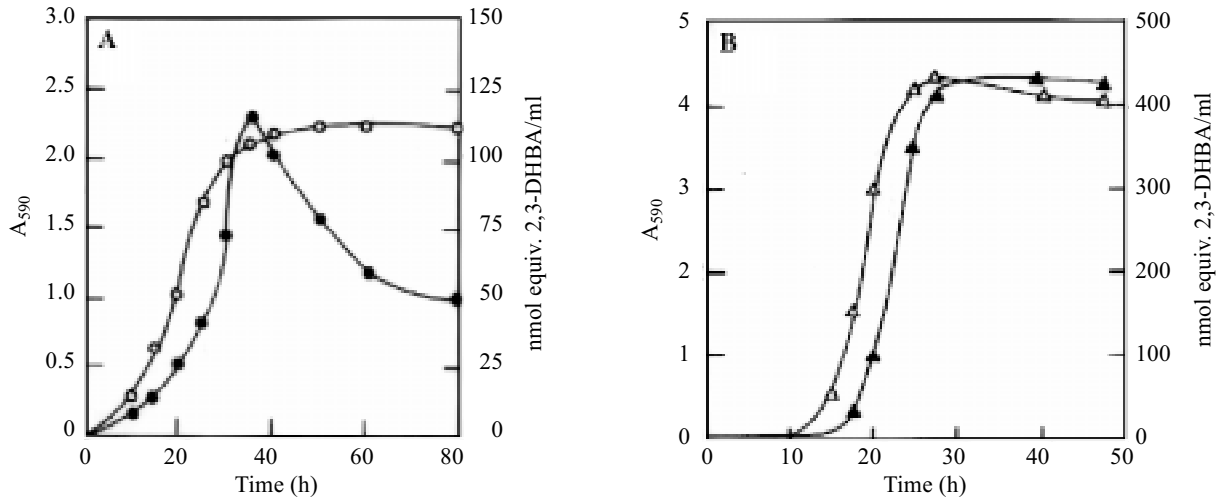

Fig. 2. Growth (○, △) and siderophore production (●, ▲) by the wild type BICC 651 (A) and the siderophore overproducing mutant N15 (B).

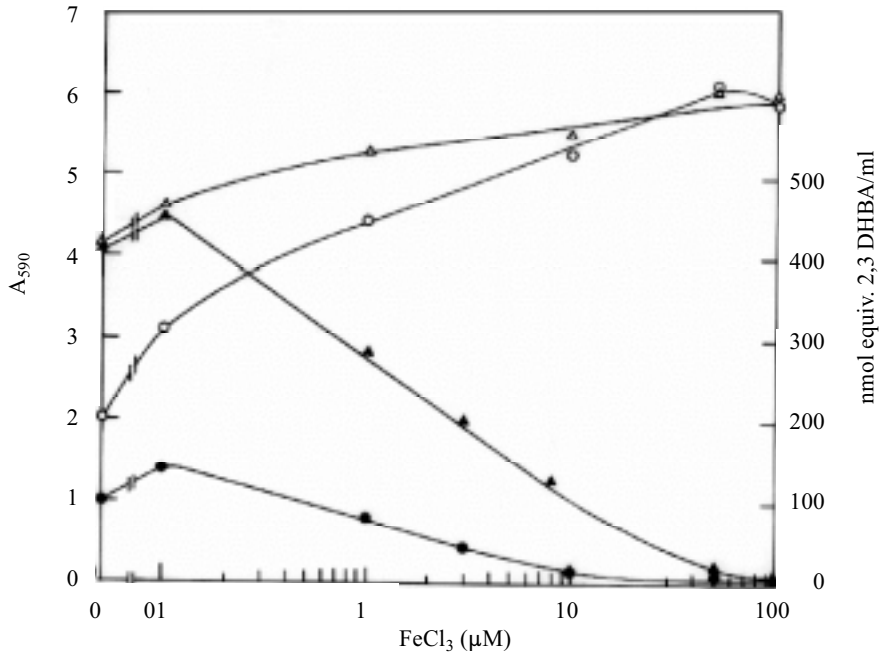

Fig. 3. Effect of increasing concentrations of iron on growth (○, Δ) and siderophore production (●, ▲) by the wild type BICC 651 (○, ●) and the siderophore overproducing mutant N15 (Δ, ▲).

651 (Table II). It was suggested that the differences between strains in nodule formation under iron-deficient conditions may be caused by their different abilities to acquire iron for nodule initiation and development (O'Hara *et al.*, 1988a). Siderophore overproducing mutants may be better adapted for iron acquisition and thus for plant nodule initiation. The observation that in nonsterile pot culture control plants without externally supplied bacterial inoculum were similar to those inoculated with the wild type suggests that the *M. ciceri* strain BICC 651 is similar to or even inferior to the indigenous rhizobia in its nodulation efficiency. However increased nodulation and greater weight of nodules per plant and increased plant growth

Table I
Symbiotic properties of *M. ciceri* wild type strain BICC 661 and siderophore overproducing mutant N15 under sterile condition*

Strain	Nodule number/plant ± SD	Dry weight of shoot/plant (g) ± SD	Acetylene reduction (nmol ethylene h^{-1} g^{-1} nodule) ± SD
Control	0	0.484 ± 0.006	0
BICC 651	9.0 ± 0.8	1.053 ± 0.032	2850 ± 25.6
N15	11.5 ± 0.8	1.401 ± 0.014	3558 ± 56.7

* Nine plants were considered for each set of treatment

Table II
Symbiotic properties of *M. ciceri* wild type strain BICC 661 and siderophore-overproducing mutant N15 under nonsterile condition in pots containing alluvial soil*

Strain	Nodule number/plant ± SD	Nodule weight/plant (mg) ± SD	Dry weight of shoot/plant (g) ± SD
Control	71.1 ± 1.6	463.76 ± 112.4	2.3 ± 0.48
BICC 651	75 ± 13.8	477.73 ± 84.9	2.3 ± 0.40
N15	101.4 ± 12.6	747.18 ± 101.0	2.9 ± 0.40

* One hundred plants were considered for each set of treatment

caused by the siderophore overproducing strain compared to plants treated with wild type bacteria reflects greater nodulation efficiency of the strain N15 than its wild type parent. Manjanatha *et al.* (1992) studied siderophore overproducing Tn5 mutants of *Sinorhizobium fredii* for their competitive abilities in an alkaline soil. According to their studies such mutants were less competitive than the wild type strain. Our data are in agreement with those of Barton *et al.* (1992) who examined *Sinorhizobium meliloti* 1021 and its Tn5 generated mutants with altered rhizobactin activities for symbiotic activities. They found a correlation between dinitrogen fixation and rhizobactin producing capability in *S. meliloti* strains. Recently, siderophorogenic bradyrhizobia have been shown to be more efficient in the nodule formation and in the promotion of soybean growth in pathogen infested soils (Khandelwal *et al.*, 2002). It would be interesting to study the potential of siderophore overproducing strains as bioinoculant for chickpea.

Literature

Arnow L.E. 1937. Colorimetric determination of the components of 3,4-dihydroxyphenylalanine-tyrosine mixtures. *J. Biol. Chem.* **118**: 531–537.

Barton L.L., F.A. Fekete, C.R. Vestor, P.R. Gill, Jr. and J.B. Neilands. 1992. Physiological characteristics of *Rhizobium meliloti* 1021 Tn5 mutants with altered rhizobactin activities. *J. Plant Nutr.* **15**: 2145–2156.

Gill P.R. and J.B. Neilands. 1989. Cloning of a genomic region required for a high-affinity iron uptake system from *Rhizobium meliloti*. *Mol. Microbiol.* **3**: 1183–1189.

Khandelwal S.R., A.V. Manwar, B.L. Chaudhari and S.B. Chincholkar. 2002. Siderophoregenic bradyrhizobia boost yield of soybean. *Appl. Biochem. Biotechnol*. **102–103**: 155–168.

Leonard L.T. 1943. A simple assembly for use in testing cultures of rhizobia. *J. Bacteriol.* **45**: 523–527.

Manjantha M.J., T.E. Loynachan and A.G. Atherly. 1992. Tn5 mutagenesis of chinese *Rhizobium fredii* for siderophore overproduction. *Soil Biol. Biochem*. **24**: 151–155.

McKnight T. 1949. Efficiency of isolates of *Rhizobium* in cowpea group with proposed additions to this group. *Queensland J. Agric. Sci.* **6**: 61–76.

Modi M., K.S. Shah and V.V. Modi. 1985. Isolation and characterization of catechol-like siderophore from cowpea *Rhizobium* RA-1. *Arch. Microbiol.* **141**: 156–158.

Neilands J.B. 1981. Microbial iron compounds. *Annu. Rev. Microbiol.* **50**: 715–731.

O'Hara G.W., M.J. Dilworth, N. Bookerd and P. Parpian. 1988a. Iron deficiency specifically limits nodule development in peanut inoculated with *Bradyrhizobium* sp. *New Phytol.* **108**: 51–57.

O'Hara G.W., A. Hartzook, R.W. Bell and J.F. Loneragan. 1988b. Response to *Bradyrhizobium* strain of peanut cultivars grown under iron stress. *J. Plant Nutr.* **11**: 6–11.

Rosenberg H. 1979. Transport of iron into bacterial cells. *Methods Enzymol.* **56**: 388–394.

Roy N., P. Bhattacharyya and P.K. Chakrabartty. 1994. Iron acquisition during growth in an iron-deficient medium by *Rhizobium* sp. isolated from *Cicer arietinum*. *Microbiology* **140**: 2811–2820.

Schwyn B. and J.B. Neilands. 1987. Universal chemical assay for the detection and determination of siderophores. *Anal. Biochem.* **160**: 47–56.

Tang C., A.D. Robson and M.J. Dilworth. 1990. The role of iron in nodulation and nitrogen fixation in *Lupinus angustifolius* L. *New Phytol.* **114**: 173–182