Tn5 induced mutants of iron assimilation pathway in *Cicer Rhizobium* strain, COBE13

Dipnarayan Saha & Aqbal Singh*
Plant-Microbe Interaction Group, NRC on Plant Biotechnology, Indian Agricultural Research Institute, New Delhi 110 012, India

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Siderophore mutants provide an understanding of the iron assimilation pathway under iron limiting conditions. Random Tn5 insertion mutants with varying phenotypes were isolated in *Cicer-Rhizobium* strain COBE13. Analysis on siderophore crossfeeding plate identified these mutants as defective in siderophore synthesis, uptake and utilization deficient in siderophore-Fe (III) complex and in regulation.

Almost all facultative, anaerobic and aerobic organisms produce extracellular siderophore to chelate iron, an essential component for growth under Fe (III) stress condition. Although voluminous information is available on the chemistry of these natural chelating agents, the genetic and molecular mechanism(s) of siderophore production, their uptake and subsequent utilization by the bacterial cell is still inadequate. In *E. coli* and *Pseudomonas* number of genes of the iron assimilation have been identified and their regulatory mechanism(s) have been understood. However, little information is available for *Rhizobium* sp. Since collection of mutants is an important prerequisite for molecular and genetic analysis in bacteria the current study was undertaken to isolate mutants of the iron assimilation pathway of *Rhizobium ciceri* strain, COBE13.

Materials and Methods

Bacterial strains—Wild type *Rhizobium ciceri* strain COBE13 (Na*, Cml*, Amp*) (Coimbatore strain, Microbiology, IARI strain collection) was used in this study. *E. coli* strain, WA803 (F met, thi*, supE, hsdR, hsdM) (NRCPP, IARI) harbouring the plasmid pGS9, (Inc N, rep 15A, Cml*, Oh*) was used as the Tn5 (Kan*) donor in biparental matings.

Culture medium—Complete TY medium was used for growing *Rhizobium* strain COBE13 and the transconjugants (WA803 X COBE13). *E. coli* strain WA803 was consistently maintained on LA agar plates.

Siderophore detection and determination medium—Siderophore production was detected on CAS-A agar medium with slight modification of the medium described by Schwyn and Neilands. A litre of CAS-A medium consists of 60.5 mg of Chrome Azurol S (CAS) dissolved in SO ml of deionized water and mixed with 10 ml of Fe*+* solution containing 1 mM FeCl₃.6H₂O, 10 mM HCl. With gentle stirring the solution was added to 72.9 ml of DEF (2% deferrated casamino acid (30%), 5 ml of deferrated α-glutamic acid (10%) and 0.5 ml of deferrated vitamin mix under continuous gentle stirring. Basal medium consists of a mixture of several salts such as 6 g of NaOH, 3.2 g of PIPES (free acid), mannitol 36.4 g, NaCl 0.2 g, NH₄Cl 0.2 g, deferrated CaCl₂ (1M) 0.4 ml, deferrated MgSO₄ .7H₂O (1M) 0.8 ml, deferrated K₂HPO₄ (2%) 10 ml and 25x micronutrient mix (NaMoO₄, 5 mg; ZnSO₄ .7H₂O, 4mg; H₃BO₃; 6.25 mg in 100 ml milipore water) 4 ml respectively in 848 ml of double distilled water. Agar @ 16 g/1000ml of total solution was added to the basal medium after the pH was adjusted to 6.8.

The growth of the mutants were further detected on low iron medium (LIM) containing mannitol 3.64 g, deferrated casamino acid (10%) 10 ml, deferrated...
MgSO₄·7H₂O (10%) 2 ml, CaCl₂·2H₂O (10%) 0.5 ml, K₂HPO₄ 0.058 g, NaCl 0.2 g and micronutrient mix 1 ml per litre. Finally 16 g of agar was added after adjusting the pH to 6.8 with HEPES (N-2-hydroxyethyl piperazine-N-2-ethanesulphonic acid) buffer (10 mM). The iron deficiency of the medium was attenuated by adding 200 µM bipyridyl (BIP), an iron chelating agent, prior to pouring of the plates. All the glasswares and medium used for siderophore studies were deferrated thoroughly.

Isolation of mutants—Tn5 induced random mutagenesis of COBE13 strain was performed as described by Selvaraj and Iyer with slight modifications. Cells of COBE13 and Tn5 donor WA803 harbouring pGS9 were grown to late log phase. Separately 1 ml of each culture was spun and suspended in 50 µl of TY broth and mixed (1:1) thoroughly. The mating mixture was patch incubated on TY agar surface for 24 hr at 30°C and the growth was scraped and suspended in 2 ml of 0.01M MgSO₄ buffer. 100 µl of this suspension was spread on TY agar plates supplemented with Kanamycin (100 µg/ml) and Nalidixic acid (50 µg/ml) and plates were incubated at 28°C for 4 days. Transconjugants (both Kanʰ & Nalʰ) were streak purified on the same kind of TY agar plates and tested on CAS-A medium for altered siderophore production. Colonies lacking orange halo and colonies with large orange halo on CAS-A medium were identified as siderophore defective (Sid⁻) and siderophore over producing mutant respectively. The relative estimation of siderophore production was carried out by taking the average area of colony growth and orange halo from 10 colonies of each mutant types (Table 1). Putative Sid⁻ derivatives that failed to grow on LIM + BIP (200µM) were confirmed as Sid⁻ mutants. All the mutants were also checked for streptomycin resistance (Str⁵).

Phenotypic complementation of mutants—Phenotypic complementation was done with cross feeding experiment in which a loopful of fresh culture of each mutants and wild type COBE13 were dispensed separately in 1 ml of sterile distilled water and streaked perpendicular to the wild type COBE13 on LIM + BIP (200 µM) medium. The streaked lines maintained a narrow gap of about 2 mm with the wild type and incubated for 4 days at 28°C and observation recorded.

Characterization of mutants for random Tn5 insertions—Genomic DNA of the wild type COBE13 and the mutant strains were isolated from 1 ml of stationary phase culture using Wizard™ Genomic DNA purification kit (Promega) and the DNA was digested with EcoRI (Promega). Fragments ran on 0.7% agarose gel in 1 x TBE buffer and were blot transferred to Hybond N⁺ membrane (Amersham Lifescience) using the alkaline blotting procedure prescribed by Amersham Lifescience.

About 25 ng of 3.3 kb Tn5 DNA purified from HindIII digestion of pGS9 DNA was labelled with (α-³²P) dCTP (BARC, Bombay) with random primer DNA labelling kit (Boehringer Mannheim). Southern hybridization of the blot transferred genomic DNA of COBE13 and mutant strains were carried out with the 3.3 kb HindIII Tn5 labelled probe. Autoradiographic (Amersham Lifescience) results of the southern hybridization were finally analysed.

Results

Tn5 insertion frequency—The transconjugants of WA803 (pGS9) X COBE13 shows both Kanʰ and Nalʰ properties whereas the parental strain COBE13 was otherwise Kanˢ and Nalʰ. The Kanʰ resistance was due to the integration of Tn5 into the COBE13 genome. The frequency of Kanʰ and Nalʰ transconjugants was 9 x 10⁵ transconjugants per recipient cell. Checking of these transconjugants on CAS-A medium for altered siderophore production led to isolation of four mutants, a mutational frequency of 0.296%.

Identification of the mutants—Among the four mutants two of them (SOPM-1 and SOPM-2) produced a large orange halo on CAS-A medium indicating over production of siderophore whereas other two (SDM-1 and SDM-2) lacked the orange halo indicating their defectiveness in siderophore biosynthesis (Fig. 1). The relative estimation of siderophore production by the strains are given in Table 1.

One of the Sid⁻ derivative (SDM-1) failed to produce any growth on LIM+BIP (200µM) medium and it was therefore considered as true defective mutant (Fig. 2).

Crossfeeding studies with mutants—In crossfeeding experiment mutant SOPM-2, SDM-1 and SDM-2 were found to grow nearest to the wild type COBE13 on LIM+BIP (200 µM) plate when streaked...
prependericularly to the COBE13 (Fig. 3). The utilization of homologous siderophore were due to phenotypic complementation. However no growth was found for SOPM-1.

**Hybridization with 3.3 kb HindIII Tn5 fragment**—The 3.3 kb HindIII fragment of Tn5 DNA

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**Fig. 1**—Screening of siderophore mutants on CAS-A medium. Fifth colony each from the left hand side of second and fourth row are siderophore defective mutants (SDM-1 and SDM-2). Third colony from the left hand side of the third row is siderophore overproducing mutant (SOPM-1). Fourth colony in the final row is wild type COBE13.

**Fig. 2**—Growth of siderophore derivatives on low iron medium (LIM). wt - wild type COBE13, a - SOPM-1 with no colony growth, b - SOPM-2 with maximum growth, c - SDM-1 lacking colony growth, d - SDM-2 with slight colony growth (leaky mutant), e and f - colony growth of randomly picked transconjugants.

**Fig. 3**—Homologous siderophore (COBE13) utilization by siderophore mutants in crossfeeding experiment on LIM + BIP (200mM) plate. wt - wild type COBE13, a - SOPM-1 lacking growth indicating its siderophore utilization defectivity, b - SDM-1, c - SDM-2 (both SDM-1 and SDM-2 are been crossed by wild type COBE13), d - SOPM-2.
Table I—Relative estimation of siderophore production by COBE13 and its mutants on CAS-A medium

<table>
<thead>
<tr>
<th>Strain</th>
<th>Area of colony growth (mm²)</th>
<th>Area of orange halo (mm²)</th>
<th>Relative magnitude of siderophore production (c=b/a)</th>
</tr>
</thead>
<tbody>
<tr>
<td>COBE139</td>
<td>4.28 (11 mm)²</td>
<td>143.06 (13.5 mm)²</td>
<td>1.50</td>
</tr>
<tr>
<td>SOPM-1</td>
<td>50.24 (8 mm)²</td>
<td>452.16 (24.0 mm)²</td>
<td>9.00</td>
</tr>
<tr>
<td>SOPM-2</td>
<td>86.55 (10.5 mm)²</td>
<td>240.40 (17.5 mm)²</td>
<td>2.78</td>
</tr>
<tr>
<td>SDM-1</td>
<td>25.95 (5.75 mm)²</td>
<td>28.26 (6.0 mm)²</td>
<td>1.09</td>
</tr>
<tr>
<td>SDM-2</td>
<td>38.46 (7 mm)²</td>
<td>50.24 (8.0 mm)²</td>
<td>1.30</td>
</tr>
</tbody>
</table>

SOPM—Siderophore over producing mutant, SDM—Siderophore defective mutant

* Values in bracket represents diameter.

Discussion

Since Tn5 induced mutagenesis have certain advantage in facilitating genetic analysis in bacterial systems, it has been employed for high affinity iron uptake mutant isolation. In Tn5 mutagenesis the suicidal nature of the pGS9 in Rhizobium leads to Tn5 integration in the genomic DNA, which imparts Kan property to the otherwise Kan COBE13. The mutational frequency was higher (0.296%) than NTG mutation (0.152%) for iron assimilation pathway of strain H68 (unpublished data).

The siderophore synthesis by the mutant SOPM-1 and SOPM-2 on CAS-A medium was more than the wild type COBE13 (Table 1) and was definitely due to their over production of siderophore. However the poor growth of SOPM-1 is indicative of its defect in siderophore - Fe (III) utilization pathway. Although lack of growth on LIM+BIP medium further supports this view, a detailed genetic analysis is needed to understand the actual nature of the mutation involved.

The overproduction of siderophore and corresponding large colony growth by SOPM-2 implies its normal siderophore-Fe (III) complex utilization but alteration in the regulation level of the genes as a consequence of the mutation. Though there is no detailed literature available about the high affinity iron assimilation pathway in Rhizobium ciceri we can make assumption of a similar system of E. coli. A protein product of fur regulatory gene represses the siderophore biosynthesis on binding to the sequences close to the RNA polymerase binding site in the promoter under high cytoplasmic Fe²⁺ condition of the cell. The increased SOPM-2 gene expression for siderophore synthesis could be explained by a mutation in the promoter region resulting in enhanced RNA polymerase binding. Such mutation would not interfere with the fur gene repression activity under high Fe³⁺ condition but the siderophore biosynthesis in the mutant(s) would be upscaled relative to the wild type. The mutation in SOPM-2 could be of such a kind and this of course need further investigation through detailed molecular genetic studies since other alternatives to explain the phenotype of the mutant do also exists.

The failure of the SDM-1 and SDM-2 strains to produce siderophore on CAS-A medium identifies them as siderophore biosynthesis defective mutants.
However the slight growth of SDM-2 on LIM could be due to its leaky nature. In crossfeeding experiment mutants SDM-1 and SDM-2 were complemented with siderophore produced by the parental strain COBE13 and hence their growth was found at the streak ends close to COBE13. The failure of mutant SOPM-1 to utilize crossfed siderophore is thus probably due to its defect in its siderophore utilization pathway.

The southern hybridization of the genomic DNA of the mutants with labelled Tn5 probe strongly concludes about the random Tn5 insertions in the genomic DNA of COBE13 leading to different types of mutants of the iron assimilation pathway.

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Reference