International Journal of Advanced Science and Research
ISSN: 2455-4227
Impact Factor: RJIF 5.12
www.allsciencejournal.com
Volume 3; Issue 2; March 2018; Page No. 61-65

Transposon Tn5-induced mutagenesis of kanamycin resistance *Rhizobium japonicum*

Bhutada SA
Department of Microbiology, Sanjivani Arts, Commerce and Science College, Kopargaon, Maharashtra, India

Abstract
Background: Transposase is an enzyme that binds to the ends of a transposon and catalyzes the movement of the transposon to another part of the genome by a cut and paste mechanism or a replicative transposition mechanism. *Rhizobium* is a genus of Gram-negative soil bacteria, many of which fix nitrogen. Nitrogen fixation is an important part of the nitrogen cycle.
Objectives: The objective of this research was to determine whether at random transposon mutagenesis could be applied in genetic studies of *Rhizobium japonicum*. One major reason for this is that a range of defined mutations is not available.
Methods: In present study *R. japonicum* at frequencies sufficient to allow the isolation of large numbers of insertion mutants. The selection of Tn5 mutants was facilitated by the expression, in all the *R. japonicum* strains we have tested, of the Tn5 encoded kanamycin resistance. A number of auxotrophic and symbiotically defective, single, random transposon (Tn5) mutants were obtained in three slow-growing strains of different DNA homology and serogroups and the single fast growing strain of *R. japonicum.*
Conclusions: In conclusion, the Tn5 can be used as a generalized mutagen to isolate a variety of mutants with defects in symbiotic nitrogen fixation. The analysis of such mutants should prove to be useful in elucidating the biochemical, genetic, and regulatory events involved in the *R. japonicum* which effectively nodulates certain Indian soybean cultivars.

Keywords: transposon tn5, kanamycin resistance, *Rhizobium japonicum*

Introduction

Transposase is an enzyme that binds to the ends of a transposon and catalyzes the movement of the transposon to another part of the genome by a cut and paste mechanism or a replicative transposition mechanism. The word "transposase" was first coined by the individuals who cloned the enzyme required for tranposition of the Tn5 transposon (Aronovich, 2011) [1]. The transposon codes for antibiotic resistance to kanamycin and other aminoglycoside antibiotics. Tn5 and other transposases are notably inactive. Because DNA transposition events are inherently mutagenic, the low activity of transposases is necessary to reduce the risk of causing a fatal mutation in the host, and thus eliminating the transposable element. Transposition is an illegitimate recombination process mediated by transposable elements such as bacterial transposons. Transposition can give rise to a variety of genome rearrangements such as insertions, deletions, inversions and chromosome fusions (Aziz *et al.,* 2010) [2].

The fundamental macromolecules involved in transposition are an element-encoded protein called transposase (Tnp) that catalyzes the multiple sequential steps in transposition and the specific DNA sequences that define the ends of the element (Berg, 1980; Duncan, 1981) [4, 8]. Transposable elements play a special role in bacterial evolution because of their ability to move between the chromosome and the various plasmid and temperate phage DNAs resident in a bacterial cell and, when piggybacked on these molecules, to move between unrelated bacteria in a population (Berg, *et al.,* 1980) [4]. *Rhizobium* is a genus of Gram-negative soil bacteria, many of which fix nitrogen. Nitrogen fixation is an important part of the nitrogen cycle. Plants cannot use atmospheric nitrogen (N_2) they must use nitrogen compounds such as nitrates. All dicotyledonous plants are generally produce effective symbiosis when nodulated by slow-growing strains of *Rhizobium japonieum* (Hahn *et al.,* 1984) [11]. While knowledge of symbiotic genes of fast-growing *Rhizobium* species has rapidly increased, relatively little progress has been made with the slow-growing species. One major reason for this is that a range of defined mutations is not available (Ludwig, 1984; Fuhrmann *et al.,* 1982) [13, 9].

Therefore, it is necessary that a random mutagenesis procedure be established for the identification and eventual isolation of the genes involved in various steps of the process of symbiosis. Transposon Tn5 mutagenesis has proved to be an effective tool in the exploration of symbiotic functions of fastgrowing rhizobia. Transposon Tn5 mutagenesis has proved to be an effective tool in the exploration of symbiotic functions of fast growing rhizobia. We demonstrated here *R. japonicum* at frequencies sufficient to allow the isolation of large numbers of insertion mutants. The selection of Tn5 mutants was faciliated by the expression, in all the *R. japonicum* strains we have tested, of the Tn5 encoded Kanamycin resistance.

Materials and Method

Tn5 transposome carries a gene that codes for kanamycin resisitance. However, this can be transferred to the sensitive strain from the donor (resistant) strain by conjugation. The transconjugants are grown on the selective media. The

bacterial cells growing on selective media contains kanamycin resistance.

Sample collection for rhizobium: Eleven Soil sample were collected from five different fields of Aurangabad.

Sample collection for *Escherichia coli*: Five Sewage sample collected for isolation of *Escherichia coli*

Bacterial strains and plasmids: The bacterial strains and plasmids used in this study are listed in Table 1. *R. jaiponicuin* and *Escherichia coli* stock cultures were maintained on agar plates supplemented with the appropriate antibiotic (ampicillin, 25, ug/ml; chloramphenicol, 25 p.g/ml).

Table 1: Bacterial strains and plasmids

Strain or Source or plasmid	Genotype or Phenotype
R. japonicum	Wild type Rifr
E. coli	tonA21 thyA6 dra-J X-
Plasmids RP4 pSUPlOll	Apr Tcr Kmr Cmr Kmr Nmr oriTRP4

Rif, rifampin; Km, kanamycin; Ap, ampicillin; Tra, transfer functions; Tc, tetracycline; Cm, chloramphenicol; Nm, neomycin; oriTRp4, origin of transfer from RP4; r, resistance; s, sensitivity.

After the collection of soil sample, these were taken and 1gm of this sample was added to 9 ml of sterile water to make 1:10 dilution, adding 1ml of the 1:10 dilution of 9ml of sterile water makes a 1:100 dilution and so on.

E. coli strain EC5 was found resistance against Kanamycin antibiotics hence it was used as the conjugal donor to introduce into *R. japonicum* (RB5) recipients. From three different matings, exconjugants were isolated on complex medium (modified 20E) containing 200 g/ml of kanamycin and streptomycin.

The physical analysis of Tn5 mutants showed that there were genuine random insertions in the *Rhizobium* genome. To show that these insertions can be correlated with phenotypic changes, we screened the Tn5 mutants for auxotrophy. The screening was done on a nitrogen-limiting minimal medium

Isolation and identification of *Rhizobium* spp: After the collection of soil sample, these were taken and 1gm of this sample was added to 9 ml of sterile water to make 1:10 dilution, adding 1ml of the 1:10 dilution of 9ml of sterile water makes a 1:100 dilution and so on. The bacterial strains isolated with the ability to degrade and performed on the basis of macroscopic and microscopic examination and biochemical test. The bacterial isolates were identified macroscopically by examining colony by examining colony morphology, surface pigment, size shape, margin, surface on media plates and microscopic examination including, grams staining to study the staining behavior, shape, and cell arrangement and granulation, spore staining. The motility test was also per performed biochemical test (Fuhrmann and Hennecke, 1982) [10].

Results and Duscussion

Isolation of *Rhizobium* from nodules *Rhizobium* was isolated from soil of various fields of Aurangabad District, which were very efficient nitrogen fixers.

Maintenance of cultures: The *Rhizobium* isolate was grown at 28^0C for 48 hrs, using YEMA medium. After growth, the cultures were stored at (5 ± 1) 0C. The growth was fast on YEMA (Yeast Extract Mannitol Agar) medium.

Morphological Characteristics: The growth of *Rhizobium* on YEMA medium produced circular, entire, convex, white, and gummy. Colonies did not absorb Congo red and they were 2-5 mm in diameter The isolate was fast growers and growth occurred in 2-3 days when incubated at 28^0C (Photo 1).

Table 2: Rhizobiam spp isolated from different location and their growth character on YEMA madiaum

Sr. No	Fields location	Isolates code	Growth on YEMA
1.	Karmala	RB1	+
2.	Mukundwadi	RB2	-
3.	Badlapur	RB3	-
4.	Valuj	RB4	-
5.	Shendra	RB5	+
6.	Kumbhephal	RB6	+
7.	Naregaon	RB7	-
8.	Chikhaldhana	RB8	+
9.	Malewada	RB9	-
10.	Takli	RB10	+
11.	Daulatabad	RB11	-

Genomic DNA isolation from *Rhizobium:* The isolated DNA was loaded in the 3 lane of gel where the ladder DNA (10 Kb) was in the first lane. The isolated DNA had high molecular weight than ladder (Fig1.)

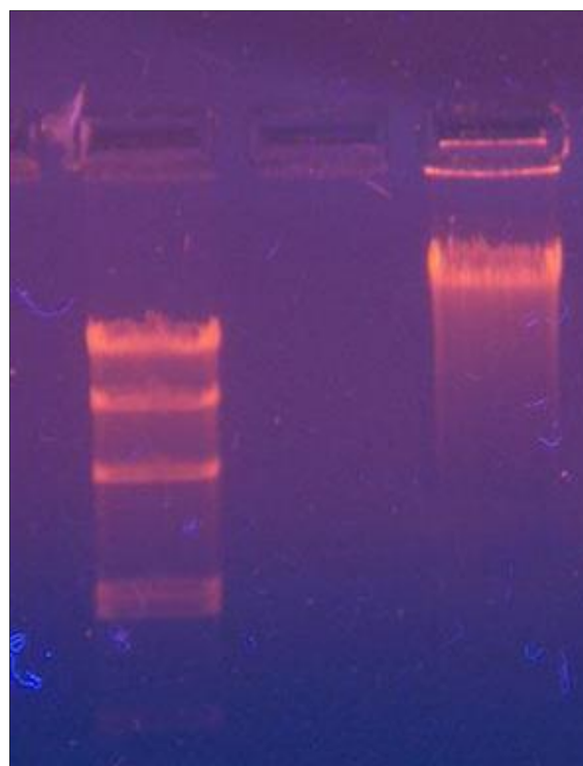

Fig 1: Extraction of Genomic DNA Lane 1: M-10 Kb Marker, Lane 2: Genomic DNA

PCR amplification of 16S rDNA: The structure of rDNA cluster and the expected amplific products (1500 bp) was showed in Fig. 4.7. The 16s rRNA regions were successfully amplified from DNA of one isolate of *Rhizobium japanicum* strain RM3166 by 16srRNA specific primers.

16S rRNA sequence of Rhizobium DNA: Based on the morphological, biochemical tests and Bergey‘s manual of Systemic Bacteriology, Vol.1, (page no. 35- 248) the given isolate was representing the genus Rhizobium. The isolates were further confirmed and characterized by conducting partial sequencing of 16S rRNA gene analysis which was carried out by NCCS (National Centre for Cell Science), Pune, India.

16S rRNA sequencing of the genomic DNA (565 bp) showed 100% similarity with species of Rhizobium meliloti strain RM3166 (Accession number: AF084539.1). The sequence was further used for BLAST analysis from NCBI data base. The 99% homology was observed with *Rhizobium spp* indicating the obtained isolate was *Rhizobium japanicum*

Phylogenetic tree: The program can be used to compute two evolutionary distances, namely the Jukes and Cantor (Jukes & Cantor, 1969) and Olsen models (Swofford & Olsen, 1990) as incorporated in the module Distance‘. The output of this module can be used for tree-making methods implemented in the PHYLIP package (Felsenstein, 1993). The phylogenetic tree was constructed (2).

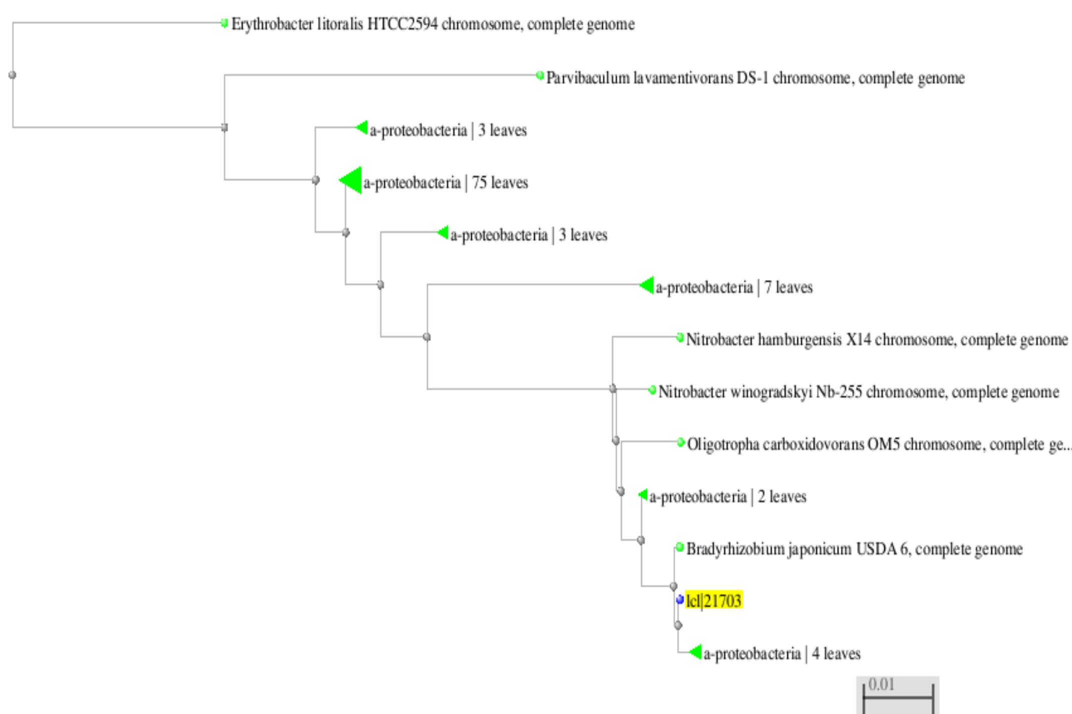

Fig 2: Phylogenetic tree analysis gene

Screening of isolates for streptomycin resistance

Eleven samples were collected from different flieds of Aurangabad. Five isolates of *Rhizobium* sp. Used for competitive studies were screened for their antibiotic resistance markers (table 1) using antibiotic disc assay method on Bergersen’s medium (Bergersen, 1961) [5]. The discs (Span Diagnostics, Surat) used were streptomycin, 10; kanamycin, 30; µg/disc.

Table 3: Antibiotic resistance spectra of different strains of Rhizobium sp. Against Streptomycin

Sr. No	Isolates code	Antibiotic spectrum
1.	RB1	S
2.	RB5	R
3.	RB6	S
4.	RB8	S
5.	RB10	S

Table 4: Biochemical identification of *E. coli*

Sr. No	Samples	Indole Test	Methyl Red Test	Voges Proskeur Test	Simmon's Test
1.	EC1	+	+	-	-
2.	EC2	+	+	-	-
3.	EC3	+	+	-	-
4.	EC4	+	+	-	-
5.	EC5	+	+	-	-

Table 5: Antibiotic resistance spectra of different strains of *E. coli* against Kanamycin

Sr. No	Isolates code	Antibiotic spectrum
1.	EC1	S
2.	EC2	S
3.	EC3	S
4.	EC4	S
5.	EC5	R

Isolation of kanamycin resistant exconjugants

E. coli strain EC5 was found resistance against Kanamycin antibiotics hence it was used as the conjugal donor to introduce into *R. japonicum* (RB5) recipients. From three different matings, exconjugants were isolated on complex medium (modified 20E) containing 200 g/ml of kanamycin and streptomycin. From single matings, 250 exconjugants were isolated. The levels of kanamycin used were above the minimal inhibitory concentrations which were 50 g/ml. In all matings, the donor and recipient cultures were also plated on selective plates and no background growth was found. Transfer frequencies for kanamycin resistance were 5 x 10 -7 for *R. japonicum.*

Isolation of Tn5 induced auxotrophic mutants

The physical analysis of Tn5 mutants showed that there were genuine random insertions in the *Rhizobium* genome. To show that these insertions can be correlated with phenotypic changes, we screened the Tn5 mutants for auxotrophy. The screening was done on a nitrogen-limiting minimal medium (Bishop's modified as described in Materials and methods). Presumptive auxotrophic mutants of *Rhizobium* were obtained at a frequency of 1.4%. Further characterization of these mutants by the Holliday scheme showed that they can be grouped into two classes.

In present study shown that Tn5 encodes streptomycin resistance which is expressed in different strains and species of Rhizobium. Tn5-specified streptomycin resistance is useful as a selectable marker for Tn5 mutagenesis of *Rhizobium* strains that are naturally resistant to kanamycin. Even for Rhizobium strains that are kanamycin sensitive, the use of streptomycin resistance as the selected marker has a practical advantage because streptomycin-sensitive *E. coli* donors of Tn5 are more effectively eliminated by streptomycin than they are by other counter-selective antibiotics such as kanamycin. We established single, random Tn5 insertions in three slow-growing and one fast growing strains of *R. japonicum*. The selection of Tn5 carrying exconjugants was facilitated by the expression in *R. japonicum,* of the Tn5 encoded streptomycin resistance gene, which concomitantly counter-selected the donor *E. coli.* It has been reported previously (Putnoky *et al.,* 1983) that Tn5 carries a streptomycin resistance gene which is not expressed in *E. coli.* Preliminary experiments showed that a Tn5 donor, only 2% of the exconjugants selected on kanamycin were also streptomycin resistant (data not shown). Selection with kanamycin and streptomycin maximally reduced the spontaneous mutant background.

In certain cases, when the selection pressure for Tn5 was maintained in transconjugants, we observed a second copy of Tn5 linked to vector sequences existing as a cointegrate within the genome. We tested the purity of these cultures by streak purification from single colonies in each case. Since cointegrates have never been observed without a true primary Tn5 insertion, this indicates that the suicide of the donor plasmid is delayed by the integration of the plasmid into a different site in the genome. However, these cointegrates can be eliminated when the mutants are grown in non-selective medium and no second copy of Tn5 remains. Such a cointegration phenomenon was observed in *R. japonicum.* We have also selected a group of Tn5 mutants at a frequency of approximately 0.2% which showed extra slow growth on rich medium and very poor growth on minimal medium. One third of these extra slow-growing colonies were found to be completely inhibited by phenylalanine. Such inhibition was not observed in wild-type cultures.

Conclusion

In conclusion, the diversity of auxotrophs detected and the isolation of symbiotic mutants suggests that transposon Tn5 can be used as a generalized mutagen to isolate a variety of mutants with defects in symbiotic nitrogen fixation. The analysis of such mutants should prove to be useful in elucidating the biochemical, genetic, and regulatory events involved in the *R. japonicum* which effectively nodulates certain Indian soybean cultivars

References

1. Aron Aronovich, EL, McIvor RS, Hackett PB. The Sleeping Beauty transposon system – A non-viral vector for gene therapy. Hum. Mol. Genet. (in press), 2011.
2. Aziz RK, Breitbart M, Edwards RA. Transposases are the most abundant, most ubiquitous genes in nature. Nucleic Acid Research. 2010; 38(13):4207-4217.
3. Berg DE. Control of gene expression by a mobile recombinational switch. Proc. Natl. Acad. Sci. U.S.A, 1980; 77:4880-4884.
4. Berg DE, Egner C, Hirschel, BJ, Howard J, Johnstrud Jorgensen LRA, Tisty TD. Insertion, excision, and inversion of Tn5. Cold Spring Harbor Symp. Quant. Biol. 1980, 45:115-123.
5. Bergersen FJ. Nitrate reductase in soybean root nodules. Biocltiniica et biopltysica acta, 1961; 52:206-207.
6. Beringer JE, Benyon JL, Buchanan-Wollaston AV, Johnston AWB. Transfer of the drug-resistance transposon Tn5 to Rhizobium. Nature, 1978; 276:633-634
7. Bishop PE, Guevara JG, Engelke FA, Evans HF. Relation between glutamine synthetase and nitrogenase activities in the symbiotic association between *Rhizobium japonicum* and *Glycine max*. Plant Physiol, 1976; 57:542-546.
8. Duncan MJ. Properties of TnS-induced carbohydrate mutants in Rhizobuin meliloti. J. Gen. Microbiol, 1981, 122:61-67.
9. Fuhrmann M, Hennecke H. Coding properties of cloned nitrogenase structural genes from *Rhizobium japonicum.* Mol Gen Genet, 1982; 187:419425.
10. Fuhrmann M, Hennecke H. Coding properties of cloned nitrogenase structural genes from *Rhizobium japonicum.* Mol Gen Genet, 1982; 187:419425.
11. Hahn M, Hennecke H. Localized mutagenesis in Rhizobium japonicum. Mol Gen Genet, 1984; 193:46-52

12. Holmes DS, Quigley M. A rapid boiling method for the preparation of bacterial plasmids. Anal Biochem, 1981; 114:193-197
13. Ludwig AL. Rhizobium free-living nitrogen fixation occurs in specialized non-growing cells. Proc Natl Acad Sci USA, 1984; 81:1566 1569
14. Putnoky P, Kiss GB, Ott I, Kondorosi A. Tn5 carries a streptomycin resistance determinant downstream from the kanamycin- resistance gene. M ol Gen Genet, 1983; 191:288-294.