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Transposon Mutagenesis for enhancement of nitrogen fixing ability of *Rhizobium japonicum* infecting *Vigna radiata*

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ABSTRACT

This study developed mutant library of 800 mutants with transposition frequency of 3.7×10^{-6}. Transposon mutagenesis is an easy and highly effective method for generating bacteria with improved characteristics and gene knockouts. The slow growing *Rhizobium japonicum* isolated from root nodules of locally grown mung bean plant. The isolated strain was subjected for transposon mutagenesis to produce 800 mutants. These 800 mutants were screened for nodulation. After screening of these 800 mutants, the100 mutants showed pink coloured nodulation to mung bean plant. Out of 100 mutants 10 were promising for nodulation and nitrogen fixation. Bacterial surface polysaccharides including Exopolysaccharides (EPS) and Lipopolysaccharides (LPS) are constituents of the bacterial cell wall. They have been reported in numerous studies as symbiotically important; defects in EPS production tend to result in arrest at the infection thread stage which is the early stage of nodulation. EPS and LPS were isolated from wild variety and mutant variety. The polysaccharides production in mutant and wild variety were compared. This study was aimed to investigate the role of EPS and LPS on nodulation and enhancement of nitrogen fixation of *Rhizobium japonicum* infecting *Vigna radiata*.

Key words : *Rhizobium japonicum, Mung bean (*Vignaradiata*), Transposon mutagenesis, Exopolysaccharides (EPS), Lipopolysaccharides (LPS)*

Introduction

Bacterial surface polysaccharides exopolysaccharides (EPS) and Lipopolysaccharides (LPS) are components of the bacterial cell wall. They have been described as symbiotically important in many studies; defects in EPS production tend to result in arrest at the infection thread phase (Wang *et al*., 1999). *Rhizobium* is a gram negative soil bacterium having ability to produce nodules on root surface of legume plants (Skorupska *et al*., 2006). This symbiosis is initiated by the exchange of signal by diverse molecules between the two partners. Among them, plants secrete flavonoids into the rhizosphere region that unregulated rhizobial genes are responsible for nodule formation (Schulze *et al*., 1998).

Lipopolysaccharides and Exopolysaccharides have special role in determing host specificity to nodule development (Neeraj *et al*., 2010). In the present study by using transposon Tn3 we produced

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mutant's library of *Bradyrhizobium japonicum* infecting *Vigna radiata* with higher and lower EPS & LPS.

Materials and Methods

Media and Cultural Conditions

Organism was isolated from locally grown root nodule of *Vigna radiata.* The isolate was identified by morphological, biochemical and physiological characteristics. Pure cultures were maintained on Yeast Extract Mannitol Agar (YEMA) slants at 4 ^{0}C and frozen in 50% glycerol at -80^{0}C. Tn3 suicide vector used here was from Bangalore GeNei, India. *E.coli* was grown on Luria Bertani (LB) medium with antibiotics ampicillin (100 µg/ml), chloramphenicol (20 µg/ml) at temperature 37 ^{0}C. The minimal basal salts (M9) medium used for screening of transconjugants was supplemented with rifampicin (100 µg/ml) and ampicillin (100 µg/ml) with pH 7.2. The antibiotics and chemicals obtained from SRL Pvt. Ltd. (India).

Bacterial Mating

The spontaneous rifampicin resistant mutants of *Rhizobium japonicum* were produced by streaking wild type culture suspension of 2.8 x 10^6 on YEMA medium containing rifampicin (100 µg/ml) and were maintained on the same medium containing rifampicin (25µg/ml).

Mating between *Rhizobium japonicum* and *E.coli* was conducted by method of (Ghatage *et al*., 2017). Donor cells were harvested by centrifugation at the late exponential growth phase and recipient cells were harvested at stationary phase. Both the donor and recipient cells were washed three times with10 mM $MgSO_4$ and then mixed at a donor-to-recipient ratio of 1:1. Here donor cell is *E.coli* and recipient cell is *Rhizobium japonicum.* Then 0.1 ml of cell suspension was transferred to a filter membrane (0.45 µm pore size; 25-mm in diameter) placed on mating medium. Controls were kept as individual filtered portions of the above cells, after incubation at 8 hrs at 30^{0}C; the filter was transferred to 2.0 ml of L.B medium, diluted and plated on appropriate selective plates.

Nodulation assay

The seeds of latika variety of mung bean were obtained and surface sterilized with 3% sodium hypochlorite. Three seeds each placed in 800 pots and overnight cultures of wild and mutant's strains were used for inoculation. Sterilized soil and water used for nodulation assay, after 30 days nodulation were observed in these pots.

Estimation of nitrogenase activity

The measurement of nitrogenase activity was carried out at Vasantdada Sugar Institute, Manjri, Br. Pune, India by using the acetylene reduction method and analyzed by Perkin Elmer Gas chromatography (with dual porpak N column of 2.0 M length) with standard flame ionization detector.

Estimation of EPS

Bacterial strain was grown in 250 ml Erlenmeyer flask containing 23 ml of the medium. The flask incubated at30 ^{0}Cfor 48 hrs on a rotary shakerat 200 rpm. The EPS production was determined from 1 ml sample taken during bacterial growth. 1 ml culture broth was centrifuged at 3500 x g and the supernatant was mixed with two volumes of chilled acetone. The crude polysaccharide developed was collected by centrifugation at 3500xg for 30 min. The EPS was washed with distilled water and acetone alternatively transferred onto a filter paper and weighed after overnight drying at 105 ^{0}C.

Estimation of LPS

LPS of cell walls measured in terms of the total heptose and glycoprotein because heptose is major constituent of the LPS. For this the method Kido *et al.,* 1990 1.5 ml of overnight culture of bacterial cell is centrifuged at 16000x g for 10 min. The bacterial pellet is suspended in 100 ml of triethylamine (TAE) buffer and mixed with 200 ml of alkaline solution congaing 3 g of SDS. 6.4 ml of 2M NaOH in 100 ml of water. The mixture is heated at 60 ^{0}C for 10 min & then mixed with phenol: chloroform (1:1 v/v) centrifuge at 16000x g for 10 min, the supernatant was mixed with 200 µl of water and 50 µl of 3M sodium acetate (pH 5.2). LPS was precipitated by adding 2 volume of ethanol. The precipitate was dissolved in 200µL of 50 mM Trishydrochloride (pH 8.0), 100 mM sodium acetate and precipitated with 2 volumes of ethanol. The final precipitation of LPS was dissolved in 50 ml of water.

Data were subjected to technique of variance (ANOVA) using medcalc computer software. The significance was calculated at 5% level of probability according to Duncan multiple range test.

Results

Rhizobium japonicum AVR was successfully isolated from *Vigna radiata* nodules and identification of same was done by morphological, biochemical and physiological characteristics. A spontaneous rifampicin resistant mutant of *Rhizobium japonicum* AVR1 was grown on YEMA medium containing 100 µg/ml rifampicin this mutant nodulates mung bean plant as wild variety. *Rhizobium japonicum* AVR 1 generated colonies of 800 transconjugate by using transposon Tn3 on M9 medium with antibiotics rifampicin (100 µg/ml) and ampicillin (100 µg/ml). The transposition frequencies obtained in mating was 3.7×10^{-6}. These 800 mutants were subjected for nodulation assay. Seeds inoculated with control inoculum showed no nodulation on root system of plant. Out of 800 mutants screened 80 gave no nodulation, 112 gave white coloured nodules, 508 showed poor nodulation and 100 mutants formed pink coloured nodules on root system of mung bean plant. Among these 100 mutants, 2 mutants were selected on the basis of nodule number, fresh and dry weight of nodules, shoot fresh and dry weight and subjected for nitrogenase activity. Mutant *Rhizobium japonicum* AVR063 was depicted significant growth of nodule along with more plant biomass when compared with wild, control and other mutant's inoculated plant. The maximum nitrogenase activity has been recorded in isolate AVR 063: : Tn3 was 19.4 µmol/hr/mg fresh weight of nodule while minimum nitrogenase activity was observed in isolate AVR1 which was 9.4 µmol/hr/mg fresh weight of nodule. Highest EPS was recorded in AVR 063:: Tn3 172.00 µg/ml greater than AVR1 91.20. LPS was also highest in mutant AVR063::Tn3 which was 146.90. µg/ml.

Discussion

We successfully developed transposon mutagenesis system for *Bradyhizobium japonicum* AVR 1 by using *E.coli* S17 harboring suicide plasmid pKo3. Exopolysaccharides appear to be essential for the early infection process. Lipopolysaccharides exhibit specific roles in the later stages of the nodulation processes such as the penetration of the infection thread (Neeraj *et al*., 2010). Several biological works highlighting importance of rhizobial surface polysaccharides in establishing effective symbiosis between *Rhizobium* and legume plant. The roles of these polysaccharides were confirmed in several non EPS producing strains *Sinorhizobium meliloti* and *Rhizobium leguminosarum* bvs. *trifolii* and *viciae*, these strains were symbiotically defective due to formation of empty or uninfected nodules on host plants. From the above results and other works, we state that rhizobial polysaccharides are necessary for establishing most specific legume and *Rhizobium* symbiosis.

Table 1. Effect of inoculation of selected rhizobial isolates on nodule number and shoot weight

Sr. No.	Isolate designation	Number of nodule/plant	Fresh weight of nodule (mg)	Nodule texture	Dry weight of nodule (mg)	EPS (µg/ml)	LPS (µg/ml)	Nitrogenase activity (µmol/hr/mg nodule)
1	AVR	14.0 ±3.55	139.9 ±3.00	Pink colour	14.5 ±1.45	103.00 ± 0.88	82.20 ± 0.57	9.61 ±0.02
2	AVR 1	12.0 ±1.52	139.3 ±3.00	Pink colour	11.8 ±1.24	116.20 ± 0.58	91.20 ± 0.52	9.4 ±0.28
3	Control	-	-	-	-			-
4	AVR03	16.0 ± 3.0	180.1 ±1.62	Pink colour	21.3 ±1.35	114.50 ± 0.40	117.56 ± 0.57	10.2 ±0.15
5	AVR07	16.0 ±2.51	159.4 ±4.34	Pink colour	16.8 ±2.66	122.20 ± .50	102.80 ± 0.61	10.5 ±0.24
6	AVR018	16.0 ±1.52	150.9 5.68	Pink colour	17.4 ±0.92	116.30 ± 0.58	113.86 ± 0.3	9.82 ±0.051
7	AVR022	15.0 ±1.52	176.0 ±1.45	Pink colour	16.1 ±2.36	88.40 ± 0.60	116.40 ± 0.60	11.2 ± 0.15
8	AVR029	19.0 ±2.81	176.0 ±1.45	Pink colour	20.8 ±2.27	102.90 ± 0.33	91.80 ± 0.32	10.9 ± 0.24
9	AVR030	14.0 ±1.73	170.0 ±1.21	Pink colour	15.0 ±1.63	122.30 ± 0.60	136.80 ± 0.32	11.2 0.15
10	AVR036	12.0 ±1.63	144.8 ±2.81	Pink colour	12.4 ±0.91	144.70 ± 1.20	131.80 ± 0.56	11.2 0.15
11	AVR040	21.0 ±2.86	183.3 ±2.69	Pink colour	24.0 ±1.31	156.00 ± 1.73	146.90 ± 1.16	12.4 ±0.1
12	AVR044	10.0 ±1.63	140.8 ±2.72	Pink colour	12.4 ±1.63	136.80 ± 0.32	102.70 ± 0.78	11.5 ±0.25
13	AVR063	22.0 ±1.73	184.3 ±2.72	Pink colour	20.4 ±6.53	172.00 ± 0.57	155.00 ± 1.73	19.4 ±0.2

(Results expressed as means ± SD for three replicates are significant at $p<0.05$; values followed by the same letter in column are not significantly different at 5% level of probability according to Duncan multiple range test)

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Conflict of Interest

There is no conflict of interest between authors & co-authors.

References

Kido, N., Ohta, M. and Kato, N. 1990. Determination of lipolysaccharides by Ethidium bromide staining afters sodium dodecyl sulphate-polyacrylamide gelelectrophoresis. L *Bacteriol.* 172 : 1145-1147.

Neeraj, Gaurav, S.S., Chatterjee, S.C. and Sachin, M.C. 2010. Nodulation Efficiency in Term of Nitrogenase Activity of *Rhizobium* Mutants and their Wild Type. 2009. *Asian Journal of Agricultural Sciences*. 1(2): 29-31.

Schulze, M., Kondorosi, E., Ratet, P., Buire, M. and Kondorosi, A. 1998. Cell and molecular biology of Rhizobium-plant interaction. *Int. Rev. Cytol*. 156: 1–75.

Skorupska, A., Janczarek, M., Marczak, M. and Krol, A.M.J. 2006. Rhizobial exopolysaccharides: genetic control and symbiotic functions. *Microb. Cell Factories.* 5: 7.

Wang, L.X., Wang, Y., Pellock, B.J. and Walker, G.C. 1999. Structural characterization of the symbiotically important low molecular- weight succinoglycan of *Sinorhizobiummeliloti*. *J. Bacteriol.* 181 : 6788-6796.