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Characterization of LPS mutants of peanut specific *Bradyrhizobium* (GN17)

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Two mutants of *Bradyrhizobium* sp. (*Arachis*) strain GN17 having altered lipopolysaccharide (LPS) composition were isolated upon random Tn5 mutagenesis to study their binding with peanut root lectin (PRA II). These mutant strains designated as GN17M1 and GN17M2 produced rough colonies and showed autoagglutination. Flow cytometric analyses indicated that strain GN17M1 bind to PRA II with highest efficiency. Both the mutants synthesized only high molecular weight lipopolysaccharides as observed by silver staining of polyacrylamide gel. The LPSs from both the mutants cross-reacted with anti-GN17 LPS, however, GN17M1 LPS showed 3 times higher cross-reactivity as detected by ELISA. Carbohydrate analysis by high performance anion exchange chromatography (HPAEC) showed that glucose was the major constituent of the purified LPS from the parent strain whereas mannose appeared as major component in the GN17M2 LPS. Equivalent amount of glucose and galactosamine with significant amount of mannose and galactose was the characteristics of the GN17M1 LPS. Purified LPS from GN17M1 and GN17M2 were respectively 17 and 10 times more potent inhibitors of PRA II activity than that of parent strain GN17. Similar binding efficiencies of the mutant LPS towards PRA II was also observed by ELISA. The results of this study indicate that the composition and the arrangement of the LPS are crucial for lectin binding.
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***Keywords:* Tn5 mutagenesis, lipopolysaccharides, lectin, high performance anion exchage chromatography**

Introduction

The legume–*Rhizobium* symbiosis involves a complex sequence of events leading to the formation of root nodules in which the symbiont fixes atmospheric nitrogen. It is believed that lectins play an important role in this symbiotic process resulting in host-specificity [1–3]. Specific attachment in *Rhizobium meliloti*–alfalfa, *Rhizobium leguminosarum* biovar *viciae*–pea, *Bradyrhizobium japonicum*–soybean symbiosis has demonstrated the specific interactions between root lectins and bacterial polysaccharides [4–6]. The lectin recognition hypothesis was reconfirmed when *B. japonicum* USDA110 induced nodules on the roots of transgenic *Lotus* carrying the soybean lectin gene [7]. In non-leguminous system, wheat germ agglutinin was reported to enhance the nitrogen fixation capacity of *Azospirillum lipoferum* [8].

In peanut plant, out of different tissue lectins [9–12] only root lectin (PRA II) is present on the surface of roots [13] and its activity is inhibited by peanut specific bradyrhizobial LPS only [14]. This lectin PRA II was demonstrated to bind to peanut specific bradyrhizobial LPS through specific sugar and the interaction is species specific [3]. High content of PRA II specific sugar in the LPS molecules isolated from these host specific microbes supported the interaction between lectin and host specific rhizobial LPS [15].

All these experimental evidences indicated that the specific recognition or attachment of bacteria to plant roots involves an interaction between the root lectin and carbohydrate receptors on the bacterial symbiont. In the present study, we describe the isolation of two mutants of peanut specific B*radyrhizobium* strain GN17 with altered carbohydrate composition in the LPS molecule. The aim of this study was to find the effect of LPS alterations on the overall properties of the bacterial outer membrane and its interaction with PRA II, and that may be important for establishing the exact role of LPS in interaction.

Materials and methods

Bacterial strains and transposon mutagenesis

The bacterial strains used in this study were *Bradyrhizobium* sp. (*Arachis*) strain GN17, *Escherichia coli* strain WA803 and *Sinorhizobium meliloti* strain Rmd201. Peanut specific

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Bradyrhizobium strain GN17 was obtained from IARI, Delhi, India; *E. coli* WA803 and *S. meliloti* Rmd201 were obtained from Prof. Randhawa (University of Roorkee, India). YEM, TY and Jensens media were purchased from HiMedia Laboratories Pvt. Ltd., India. Rhizobia were grown either in YEM [16], TY [17] or Jensens medium [18] at 30°C whereas *E. coli* was grown in TY medium at 37°C. Antibiotics were supplemented as required at following concentrations: streptomycin 100 μg/ml for *Bradyrhizobium* sp. and neomycin 40 μg/ml for *E. coli*. *Bradyrhizobium* strain GN17 was confirmed on the basis of partial sequence analysis of the 16S rRNA gene and the sequence was submitted to GenBank (accession number AF333076). Tn5 mutagenesis of *Bradyrhizobium* sp. (*Arachis*) strain GN17 was carried out by the method of Selvaraj and Iyer [19]. The parent strain was crossed with *E. coli* WA803 containing Tn5 on the plasmid pGS9. Conjugation was performed by mixing strains WA803 and GN17 in the ratio of 10:1 on TY agar plates followed by incubation at 30°C for 24 h. Bacteria were suspended in TY broth and plated in appropriate dilutions on TY agar plates, supplemented with neomycin 400 μg/ml and streptomycin 100 μg/ml. Neomycin resistant (Neo^r) GN17 transconjugants were then screened after 5 days of incubation at 30°C.

Screening of mutants

Around 800 mutants were screened after transconjugation. The colonies showing non-mucoid and smaller in size were considered assuming probable association with LPS alteration. Out of 5 such colonies, two mutant strains designated as GN17M1 and GN17M2 were selected. Growth characteristics in liquid TY medium of the parent strain and the mutants were also monitored. Insertion of Tn5 was verified by PCR amplification of a 818 bp product, encoded by a region 1533–2350 bp in Tn5. The genomic DNAs of *Bradyrhizobium* strains were isolated following the method of Wilson et al. [20]. Amplification was performed by a Minicycler (MJ Research, USA) for 25 cycles (94°C–30 s, 56°C—45 s, 72°C—1 min 30 s) using genomic DNA as template and a pair of oligonucleotide primers 5′-GGATGAGGATCGTTTCGCAT-3′ and 5′-CCCGCTCAGAAGAACTCGTC-3′. The PCR products from the parent strain and both the mutants were then analyzed on 0.8% (w/v) agarose gels.

Purification of PRAII and labeling with FITC

Peanut root agglutinin (PRA II), isolated from 7 days old roots of peanut plant, was purified by affinity chromatography on Sephadex G-50 column at room temperature as described earlier [13]. Protein estimation of the purified fractions was done following the method of Lowry et al. [21]. Labeling of PRA II with FITC was performed following the method of Fabre et al. [22]. Briefly, 1 mg of purified PRA II was mixed with 100 μg of FITC (Sigma, St. Louis, MO, USA) in 0.5 M carbonate buffer (pH 9.5). After incubation for 1 h at room temperature, the mixture was loaded on to a Sephadex G-25 column (18 cm × 1.0 cm, 12.5 ml bed volume). FITC—labeled PRA II was separated from free FITC molecules by using 0.5 M carbonate buffer as eluant with a flow rate of 4 ml/min. Protein positive fractions were dialyzed against PBS (10 mM, pH 7.2) and kept at 4°C till further use.

Flow cytometric analyses

The parent strain GN17 and the mutants GN17M1 and GN17M2 were evaluated for their ability to bind to PRA II using flow cytometer (Becton & Dickinson, USA). Around 1.3×10^8 cfu/ml cells were washed thrice in PBS (10 mM, pH 7.2) and were incubated with 50 μg/ml of PRAII—FITC conjugate for 30 min at room temperature. After proper dilution with PBS, samples were read by flow cytometry. For each analysis 10,000 events were collected and the histograms were generated using Cell-Quest programme.

Isolation, purification and SDS-PAGE analysis of LPS

LPSs from the strains GN17, GN17M1, GN17M2 and Rmd201 were isolated by hot water-phenol method [23]. The water layer containing the LPS was treated with DNase I (1000 kunitz/ mg) and RNase A (100 kunitz/ mg) and stirred overnight to remove ribose sugars. The sample was then treated with proteinase K (100 μg/ml) at 37°C for 1 h. The sample was dialyzed and lyophilized. The lyophilized powder was designated as crude LPS and stored at 4°C till further use.

To remove the contaminating K antigens, the lipopolysaccharides were purified by polymyxin-agarose affinity chromatography (Detoxi-Gel; Pierce Chemical Co., USA) as described [24]. SDS-PAGE (13%) was performed with the purified LPS samples (40 μg of hexose) and the gel was stained by silver staining [25].

LPS immunoreactivity

Antibodies to LPS (strain GN17) were raised in rabbits according to the immunization protocol as described earlier [3] by subcutaneous injections of LPS (200 μg in 0.9% saline/injection). The immunocharacterization of LPS was carried out by ELISA and immunoblottting.

(a) *ELISA*. The ability of GN17M1 and GN17M2 LPSs to bind to anti-GN17 LPS was estimated by enzyme-linked immunosorbent assay (ELISA). The LPSs from the strains GN17, GN17M1, GN17M2 and Rmd201 were coated at a concentration of 10 μg/100 μl, in triplicates, in a 96 well microtitre plate (Nunc, Denmark) and allowed to incubate for overnight at 37°C. The wells were washed five times with PT buffer (10 mM PBS, pH 7.2 with 0.02% Tween 20) followed by blocking with 100 μl of 3% gelatin in PT and incubated for 1 h at 28°C. This step was followed by three consecutive washes with PT and 100 μl of 1:400 diluted anti-GN17

LPS antisera was added and allowed to incubate for 3 h at 28°C. After incubation, the plate was washed thrice with PT and 100 μl of 1:1000 diluted protein A-HRP conjugate was added and incubated at 28°C for 45 min. After washing thrice, the immunocomplexes were detected and quantified by adding 100 μl of the substrate o-phenylene diamine (1 mg/ml) containing hydrogen peroxide (5 μl/ml) in citrate phosphate buffer (0.05 M, pH 5.0). The colour was allowed to develop for 15 min at room temperature. The reaction was stopped by adding 50 μl of 3 M $H_2SO_4$to each well. The absorbance was read at 492 nm in an ELISA reader (Spectra Max 190, Molecular Devices, USA).

(b) *Immunoblotting*. For identification of LPS subfractions that supported a positive reaction in ELISA, the samples (40 μg of hexose) were electrophoretically fractionated by SDS-PAGE (13%) and transferred to nitrocellulose paper. After transfer, the membrane was incubated with 1.5% BSA in PT buffer (10 mM PBS, pH 7.2 with 0.02% Tween 20) for 2 h. This was followed by washing thrice in PT buffer, the membrane was then incubated with anti-GN17 LPS antisera (1:400) for 3 h. After washing, 1:1000 anti-rabbit IgG raised in goat was added and incubated for 1 h. The membrane was finally developed using 3, 3′-diaminobenzidine (DAB) for colour development.

Carbohydrate analysis

LPS samples (50 μg of hexose) were subjected to hydrolysis with 1% acetic acid at 100°C for 1 h and centrifuged at 10,000 × g to remove the precipitates. The oligosaccharides in the supernatant were hydrolyzed with 8 M trifluoroacetic acid (TFA, Sigma, St. Louis, MO, and USA) for 16 h as described earlier [15]. High performance anion exchange chromatography (HPAEC) was performed on a Dionex DX 500 (Dionex, Sunnyvale, CA) with a Carbopac PA 1 column (4 × 250 mm) using pulsed amperometric detector with a gold working electrode. The waveform used for the analysis was as follows: 0.05 V for 0.4 s, 0.065 V for 0.2 s and −0.15 V for 0.4 s. Prior to injection of each sample the column was washed with 200 mM NaOH and pre-equilibrated with 18 mM NaOH. The flow rate was 1 ml/min. The samples were eluted isocratically with 18 mM NaOH and 6-O-Methyl-D-galactopyranose (25 μm) was used as an internal standard.

Hemagglutination inhibition assay

The LPS from the strains GN17, GN17M1, GN17M2 and Rmd201 were allowed to incubate with purified PRA II having 4HA units for 10 min at room temperature. The samples were then mixed with 4% rabbit erythrocyte suspension in 0.01 M Tris-HCl (pH 7.2), 0.15 M NaCl, 1.0 mM $MnCl_2$ and agglutination was measured after 20 min at room temperature. Inhibition was accounted for those samples, which showed only 50% activity, after 20 min of incubation.

Lectin binding experiment by ELISA using PRA II antibodies

Antibody to PRA II was raised in rabbits in accordance to the immunization protocol as described earlier [3] by subcutaneous injection of PRA II (200 μg in 0.9% saline/injection).

The LPS (10 μg/100 μl) was coated in a 96 well microtitre plate (Nunc, Denmark) and allowed to incubate overnight at 37°C. The wells were thereafter washed five times with PT buffer (10 mM PBS, pH 7.2 with 0.02% Tween 20) followed by blocking with 100 μl of 3% gelatin in PT and incubated for 1 h at 28°C. After washing with PT, 100 μl of purified PRAII (1 μg/well) was added to the wells and incubated for 2 h at 28°C. Anti-PRA II antisera (100 μl, 1:400) was added to the wells after washing, followed by incubation for 3 h at 28°C. The plate was then washed thrice with PT and 100 μl of 1:1000 diluted protein A—HRP conjugate was added and incubated at 28°C for 45 min. After proper washing, the immunocomplexes were quantified by adding 100 μl of the substrate o-phenylene diamine (1 mg/ml) containing hydrogen peroxide (5 μl/ml) in 0.05 M citrate phosphate buffer (pH 5.0). The colour was allowed to develop for 15 min at room temperature. The reaction was stopped by adding 50 μl of 3 M H_2SO_4 to each well. The absorbance was read at 492 nm in an ELISA reader (Spectra Max 190, Molecular Devices, USA).

Results

Isolation of mutants

Mutants of GN17 obtained by random Tn5 mutagenesis using the "suicide" plasmid pGS9 were isolated as described in materials and methods. Two mutants designated as GN17M1 and GN17M2 showed rough colonies on agar plate. In liquid TY medium, both the mutants exhibited autoagglutination (Figure 1). Tn5 insertion was found only in GN17M1 (lane 2, Figure 2) and no product was amplified with the sample from GN17M2 (data not shown). The amplified product corresponded to the 818 bp product from pGS9 (lane 1, Figure 2) was considered as control.

Binding of PRA II to *Bradyrhizobium* GN17and its mutants

Interaction of FITC—labeled PRA II with *Bradyrhizobium* sp. (*Arachis*) strain GN17 and its two mutants was studied by flow cytometry. Figure 3, represents the lectin binding profiles to the rhizobial cells. There was a marked difference in binding of PRA II to the parent strain (Figure 3A) and the mutant GN17M1 (Figure 3B), wherein the total cell population (89%) took up high fluorescence. In the case of mutant GN17M2, the profile was similar to that of the parent strain (Figure 3C). A second population of cells (50–55%), which fluoresced with low intensity, was found in the strains GN17 and GN17M2 (M1 region). Differential binding of PRA II with these mutants, rough colony morphology and autoagglutination indicated possible alteration in cell surface characteristics of these cells. As LPS molecules

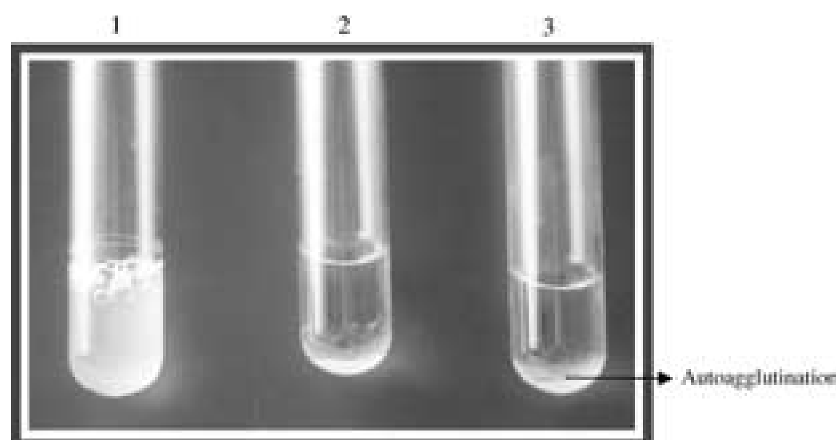

Figure 1. Growth characteristics of parent strain GN17 and its mutants in liquid TY medium. 1: parent strain GN17; 2: mutant GN17M1; and 3: mutant GN17M2.

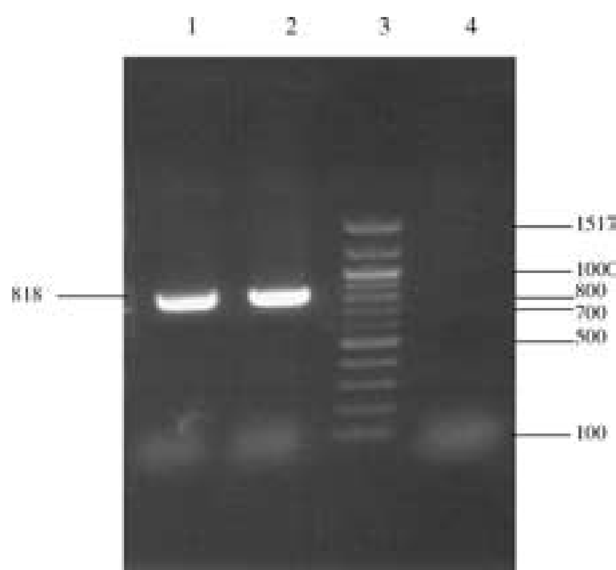

Figure 2. Verification of Tn5 insertion by PCR amplification of 818 bp product from genomic DNA of *Bradyrhizobium* sp. (*Arachis*) strains. Lane 1: *E. coli* WA803 (pGS9); lane 2: mutant GN17M1; lane 3: 100 bp ladder; and lane 4: parent strain GN17.

are very crucial for such changes therefore LPSs were purified and characterized to study their interaction with root lectin.

Analysis of purified LPS by SDS-PAGE

LPSs isolated from the strains were purified separately on Detoxi gel and were analyzed by SDS-PAGE. Figure 4 shows the silver stained banding patterns of purified LPS. According to criteria already applied to other LPS [26], components in the gel were designated as LPS I/LPS Ia and LPS II according to their electrophoretic mobilities. The parent strain (lane 1) showed ladder type of banding pattern with higher amounts of LPS II as evidenced by more intense bands, which migrated faster to the anode. The mutant LPSs (lanes 2 and 3) from both the strains GN17M1 and GN17M2, respectively, lost the bands corresponding to the component LPS II but showed prominent bands in the region marked as LPS I/LPSIa. Marked difference among silver-stained patterns between the two mutants were also observed. A band marked LPS I with slower mobility stained more in the LPS isolated from the mutant GN17M1 only.

Immunocharacterization of LPS

Immunocharacterization was carried out by ELISA and immunoblotting using antibody raised against GN17 LPS. In ELISA experiment, identical amount of LPS from all the strains was coated on the wells. LPS from mutant GN17M1 showed 3 times higher reactivity (bar 2, Figure 5) towards the anti-GN17 LPS in comparison to the parent strain LPS (bar 1). However, the LPS from mutant GN17M2 showed similar cross reactivity (bar 3) as the parent LPS. The LPS from Rmd201 did not show affinity for the anti-GN17 LPS antibody (bar 4).

When the LPS samples were analyzed by immunoblotting, it was observed that anti-GN17 LPS showed high reactivity towards high molecular weight molecules present in mutants GN17M1 and GN17M2 (lanes 3 and 2, Figure 6). However, with GN17M1 LPS the strongest signals were observed on two high molecular weight bands and this was also reflected in ELISA experiments showing higher reactivity with the same LPS sample (Figure 5). Cross reactivity of antisera with the LPS samples from GN17 (Lane 1) was found to be distributed evenly throughout the whole lane. The sample from Rmd201 was completely non-reactive (lane 4).

Carbohydrate analysis

The monosaccharide composition of the TFA hydrolysate of the purified LPS for all the three strains is presented in Table 1. Glucose was the major component with negligible amount of mannose (Figure 7A) in parent strain LPS, whereas the amount

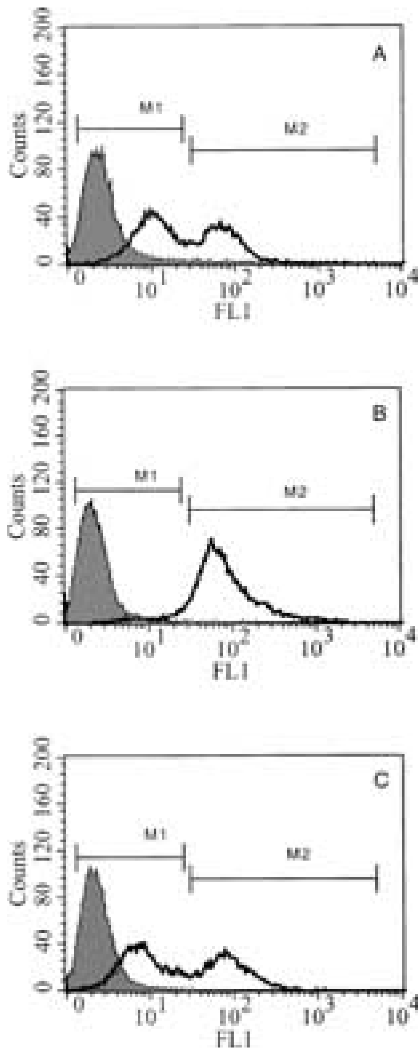

Figure 3. Flow cytometric profiles of PRA II binding to *Bradyrhizobium* sp. (*Arachis*) strain GN17 and its two mutants. The filled histograms show cells before incubation with FITC—PRA II conjugate and open histograms represent cells incubated with FITC—PRA II conjugate. Area M1 and M2 indicate low and high fluorescence, respectively. The results shown are representative of four separate experiments. Panel A—parent strain GN17; Panel B—mutant GN17M1; Panel C—mutant GN17M2.

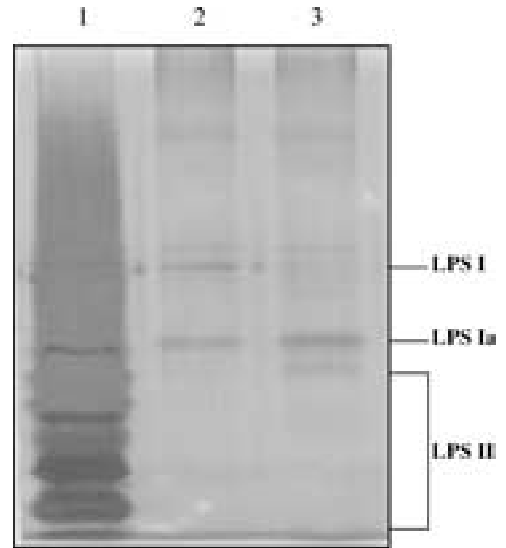

Figure 4. SDS-PAGE (13%) profile of purified LPS fractions from *Bradyrhizobium* strains. Lane 1: parent strain GN17; lane 2: mutant GN17M1 and 3: mutant GN17M2.

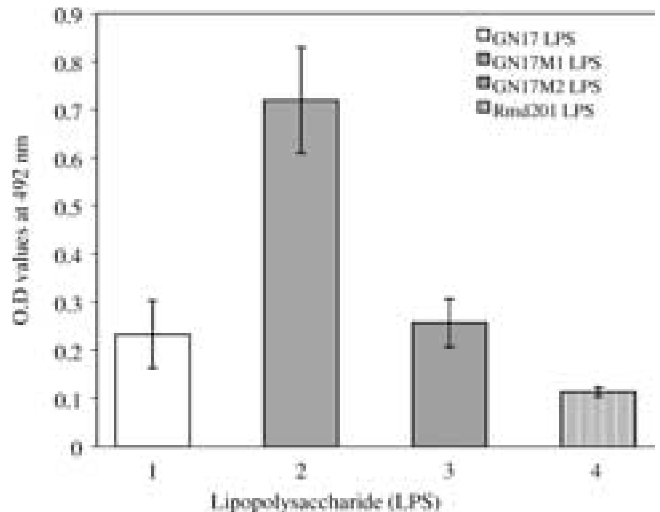

Figure 5. Immunocharacterization of LPS by ELISA using anti-GN17 LPS antibody. Bars 1, 2, 3, and 4 represent the binding of the LPS from strains GN17, GN17M1, GN17M2 and Rmd201, respectively, to the antisera.

of mannose was found to be double to that of glucose (Table 1) in the purified LPS from GN17M2 (Figure 7C). However, this glucose: mannose ratio changed to 4 in the case of LPS from GN17M1 (Figure 7B). Presence of equivalent amount of galactosamine and glucose with significant amount of galactose are some other alterations found in the monosaccharide composition of GN17M1 LPS.

Binding of LPS with PRA II by hemagglutination inhibition assays and ELISA

Hemagglutination of rabbit erythrocytes by PRA II was inhibited by LPS isolated from all three strains of *Bradyrhizobium* sp. (Table 2). Results indicated that LPS from GN17M1 was the most potent inhibitor among all the samples followed by the LPS from GN17M2. Inhibitory potency was increased by 17 and 10 fold by GN17M1 LPS and GN17M2 LPS respectively over the parent LPS.

ELISA using PRA II antibody as described in materials and methods confirmed the inhibition results. Under the experimental conditions, the ELISA was highly sensitive, detecting 100 ng/well of LPS. As presented in Figure 8, LPS from both the strains GN17M1 and GN17M2 (bars 2 and 3) showed high reactivity towards PRA II in comparison to the parent strain

Table 1. Monosaccharide composition of purified lipopolysaccharides from *Bradyrhizobium* sp. (*Arachis*) strain GN17 and its mutants

	Molar ratios of monosaccharides[a]			
Monosaccharide	*RT[b] (min)*	*GN17*	*GN17M1*	*GN17M2*
Unknown	3.70	ND[c]	ND	ND
Rham	7.20	–	–	–
GalN	7.73	0.33	3.23	–
GlcN	9.12	–	–	0.08
Gal	9.52	0.12	0.91	–
Glc	10.44	6.35	3.28	0.64
Man	11.50	0.06	0.88	1.22
Rib	14.92	–	0.90	–

[a]Molar ratio was calculated based on the internal standard (6-O-Methyl-D-galactopyranose) = 4.0.
[b]Retention time.
[c]Not determined.

Table 2. Hemagglutination inhibition assays of peanut root agglutinin (PRA II) with different lipopolysaccharides (LPS)

Strains	*Concentration of LPS[a] (μg/ml)*
GN17	1100.00
GN17M1	64.00
GN17M2	102.00
Rmd201	–[b]

[a]Minimal concentration required for 50% inhibition of 4HA units of purified peanut root agglutinin.
[b]Non-inhibitory at the concentration 3300 μg/ml.

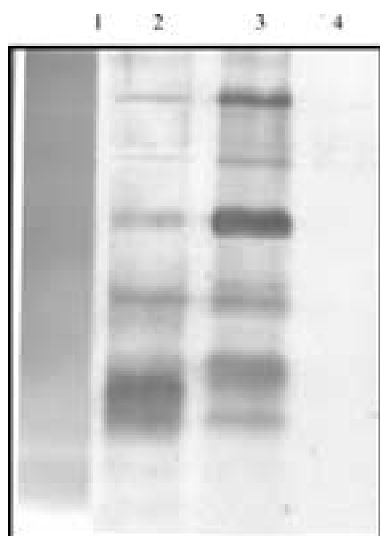

Figure 6. Western blot analysis of different LPS using anti-GN17 LPS antibody. Lane 1: parent strain GN17; lane 2: mutant GN17M2; lane 3: mutant GN17M1; and lane 4: *S. meliloti* Rmd201. Samples were loaded on to the gel based on 40 μg hexose content in each sample.

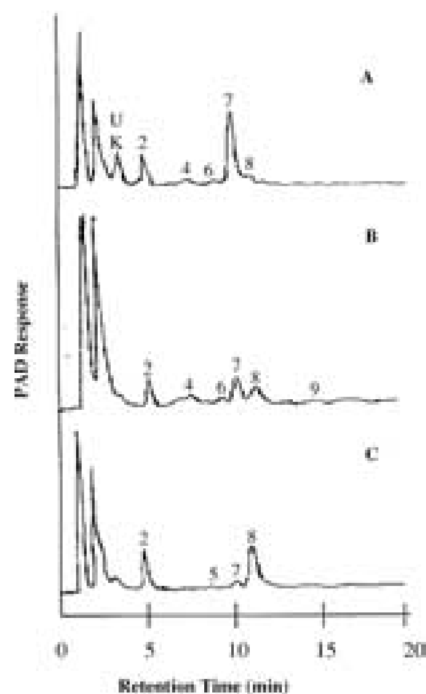

Figure 7. High performance anion exchange chromatograms for hydrolysates (2M TFA, 16 h, 100°C) of polysaccharides of LPS from parent strain GN17 (A) and its mutants GN17M1 (B) and GN17M2 (C). The identity of the monosaccharides is as follows: 2-6-O-Me-Gal (internal standard); 4: GalN; 5: GlcN; 6: Gal; 7: Glc; 8: Man; 9: Rib; and UK: Unknown.

LPS (bar 1). The LPS from *S. meliloti* Rmd201 did not show any binding to PRA II (bar 4).

Discussion

This study describes the isolation and characteristics of two *Bradyrhizobium* sp. (*Arachis*) strain GN17 mutants, which were altered in LPS synthesis. Both the mutants were obtained by random Tn5 mutagenesis using suicide vector pGS9. However, analysis of the mutants revealed that mutant GN17M1 contains Tn5 insertion (Figure 2) and mutant GN17M2 was a spontaneous neomycin resistant mutant. Isolation of LPS mutants of different rhizobial strains were reported by many groups [26–28] and frequency of occurrence of spontaneous kanamycin resistant mutants of peanut specific strain NC92 was recorded very high (1 mutant in 10^5–10^6 kanamycin sensitive cells) after pGS9 mutagenesis [20].

Usually less mucoid colonies obtained by Tn5 mutagenesis are found to have defect in LPS molecules [26,27,29]. In the present study, both the mutants display rough colony morphology and showed tendency to autoagglutinate in liquid cultures (Figure 1). Alteration in carbohydrate production is also correlated with autoagglutinating behavior of a number of LPS mutants [27]. Flow cytometric analyses of lectin binding to three strains are influenced by the cell surface carbohydrate

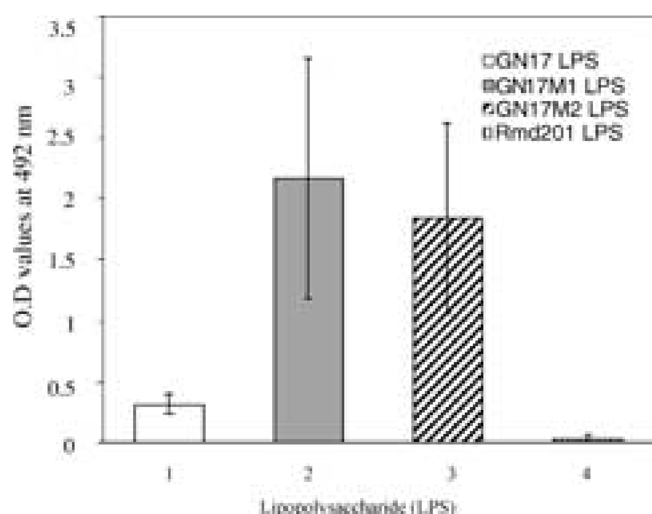

Figure 8. Binding of LPS to PRA II using anti-PRA II antibody by ELISA. Bars 1, 2, 3, and 4 represent the binding of PRA II to LPS from strains GN17, GN17M1, GN17M2, and Rmd201, respectively.

composition as well as their arrangement on the surface. This is evident from differential binding of lectin to parent and GN17M1 mutant and similar binding to parent and GN17M2 mutant. Similar profile of lectin binding to the parent (GN17) and the mutant GN17M2 was interesting despite of the variation observed in carbohydrate composition (Figure 7) of LPS population. The highly fluoresced homogeneous population (89%) of GN17M1 indicated that almost all the cells were able to bind to FITC—PRA II conjugate with same affinity. This may be corroborated to the homogeneity in the distribution of surface receptors. Two distinct fluoresced populations in the parent and GN17M2 may be linked to the heterogeneity in the surface polysaccharides. Lectin binding not only depends on the availability of the receptors on the surface but it is influenced by the other macromolecules present in the vicinity of the receptors also. The flow cytometry results may imply that the Tn5 inserted mutant GN17M1 is an improved strain over other strains with respect to lectin binding. Such improved strains of *Rhizobium trifolii* 0403 and *Sinorhizobium fredii* strain USDA 208 were reported by Tn5 mutagenesis [30,31]. Alterations in LPS have been studied in *Salmonella enterica* serovar Typhimurium and *Neisseria gonorrhoeae* using mannose binding lectin (MBL) [32]. In this study, different binding profiles of mutant strains to PRA II may also be related to the alteration in the structure and conformation of LPS molecules present on the surface of these cells. To verify any alteration in the LPS characteristics, purified samples were analyzed by SDS-PAGE.

On SDS-PAGE, purified LPS samples indicated clear differences in their electrophoretic mobility. Both the mutants showed high molecular weight LPS bands only; however, mutant GN17M1 had a more intense LPS I band (Figure 4, lane 2). The sample from the parent strain had significant amounts of low molecular weight bands (LPS II), which were not detectable in the mutants.

To assess the possible alteration in the LPS structure immunocross reactivity experiments were performed. Results from ELISA indicated that LPS from both the mutants cross reacted with anti-GN17 LPS, but the reactivity with GN17M1 LPS was 3 times more than to its parent LPS. Immunoblot experiment indicated that the anti-GN17 LPS was more reactive to high molecular weight bands (Figure 6, lanes 2 and 3) of the mutants. More intensity of these bands in mutants LPS may imply that antibodies were developed in high titer against high molecular weight bands of parent LPS. Immunocharacterization has also been used in other *Rhizobium* sp. to verify the changes in LPS molecules [26,33].

To gain further insight regarding the alteration in the LPS molecules, the monosaccharide compositional analysis was carried out. It was found that the ratio of glucose to mannose varied in all the three strains. This ratio was important since PRA II is a glucose/mannose specific lectin. The presence of glucose to mannose in the ratio of 4 in the LPS from the improved strain GN17M1 may be responsible in enhancing the lectin binding. However, true receptors for lectins are the oligosaccharides and effective lectin binding is dependent not only on cell surface carbohydrate composition but depends on the structural arrangement of the saccharides also.

The binding of PRA II to the rhizobial surface polysaccharides (LPS) is thought to be a determining factor for host specific symbiosis. We had previously shown that the LPS-recognition by corresponding host lectin was species specific as well as tissue specific and the true ligands for the root lectins are the cell wall LPS [3]. In continuance to our earlier work, the role of LPS was reevaluated. The binding of PRA II to the whole cells from mutant strains were different from each other as seen by FACS analysis but the purified LPS from both the mutants showed similar binding potency to PRA II. The discrepancy involving better lectin binding to isolated LPS from GN17M2 compared with whole-cells binding by FACS may indicate a different arrangement of LPS molecules on the surface of intact cells. Despite the fact that the rough colony rhizobial strains lack high molecular weight bands, which comprise of O-chain LPS [29], in the present study we found that the rough colony mutants GN17M1 and GN17M2 made only high molecular weight bands.

The results of the present study imply that the lipopolysaccharides of both the mutants were altered. Such alterations lead to change in rhizobial surface characteristics such as colony morphology, autoagglutination as well as binding behavior of the cells to their receptors (lectin). High molecular weight LPS, which bears the O-chain is the probable candidate ligand for lectin binding. However, the composition of the polysaccharides and their arrangement on cell surface are crucial for differential lectin binding. Future experiments will be performed to isolate and identify the gene with Tn5 insertion from these LPS mutants.

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