Role of Bacterial Polysaccharides in the Adsorption Process of the *Rhizobium*-Pea Symbiosis

Goro KATO, Yoshiharu MARUYAMA and Michinori NAKAMURA*

Department of Agricultural Chemistry, Faculty of Agriculture,
The University of Tokyo, Bunkyo-ku, Tokyo 113, Japan

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Phase-contrast and fluorescence microscopy observations showed that pea symbiont *R. leguminosarum* adsorbed to pea root hairs, but non-symbiont rhizobial strains only adsorbed to a small extent. ^{14}C -labeled cells were used to assay the number of rhizobial cells adsorbed to a pea root. Capsular polysaccharides or lipopolysaccharides obtained from *R. leguminosarum* specifically inhibited the adsorption of ^{14}C -*R. leguminosarum* cells to a pea root and specifically adsorbed to pea root hairs. Also, they reacted specifically with pea seed lectins. These results suggest that capsular polysaccharides or lipopolysaccharides play an important role in host-specific adsorption. The interaction between the polysaccharides and pea lectins could be the key to determining host specificity in the infection process of *Rhizobium*-pea symbiosis.

It is generally thought that the host specific nodulating activity of *Rhizobium* is the overall expression of the complicated root nodule formation process.¹⁾ Many researchers have studied the mechanism of the determination of this specificity. Since it was indicated that the host specificity in *Rhizobium*-clover association was revealed in the initial infection process (infection thread formation and root hair curling),²⁾ adsorption or invasion of *Rhizobium* into root hair cells has drawn attention as the determinant of host specificity. On the other hand, several reports showed that host-*Rhizobium* recognition might be based on the interaction between leguminous lectins and rhizobial cells in the infection process.^{3~10)} The lectin hypothesis in *Rhizobium*-clover symbiosis has been supported by some experimental results presented by Dazzo *et al.*^{11~15)} However, there was not enough evidence, in other host-symbiont systems, to show the host specific infection of *Rhizobium*, and the recognition reaction between plant lectin and bacterial cell surface polysaccharides in this infection stage.^{16~19)} We showed previously, that soy-

bean seed lectin and pea seed lectin bind specifically to respective symbionts, and the lectin-receptor of rhizobial cells, existed in the cell surface polysaccharide.¹⁰⁾ This paper deals with (a) the specificity in adsorption of rhizobial cells to pea root hairs; (b) the effect of the rhizobial cell surface polysaccharides on the adsorption; (c) the ability of polysaccharides to adsorb to root hairs; (d) the interaction between the polysaccharides and pea lectin; (e) the immunological analysis of the polysaccharides.

MATERIALS AND METHODS

Rhizobial strains. *Rhizobium japonicum* strains 509, 5033 and 501, *R. leguminosarum* strains J357 and J2080 and *R. meliloti* strain 105 were obtained through the courtesy of Dr. Tsuru, National Institute of Agricultural Sciences. *R. japonicum* strain J10 and *R. meliloti* strains J7017 and J702 were obtained from the Federation of Agricultural Cooperative Unions of Tokachi. *R. phaseoli* strain 7P was isolated from a nodule of kidney bean in our laboratory.

Legume seeds. Pea (*Pisum sativum*, var. Sanjūnichi-kinuzaya), soybean (*Glycine max*, var. Shiratori), alfalfa (*Medicago sativa*, var. Dupuit) and kidney bean (*Phaseolus vulgaris*, var. Satsuki-Midori No. 2) were purchased from Takii Seeds Co., Tokyo.

* Present address. Nōdai Research Institute, Tokyo University of Agriculture, Setagaya-ku, Tokyo.

The following procedures were carried out as described previously:¹⁰⁾ *maintenance and cultivation of the bacteria; test for nodulating activity of Rhizobium; phenol extraction of crude LPS; extraction and purification of pea seed lectin; assay of hemagglutinating activity of lectin; SDS-polyacrylamide gel electrophoresis of LPS and polyacrylamide gel electrophoresis of lectin; gel filtration on Sepharose 6B or 4B; determination of total carbohydrate, nucleic acid, protein and KDO.*

Observation of adsorption of Rhizobium cells to pea root hairs.

Phase-contrast microscopy. Rhizobial cultures in the late log phase, diluted doubly with 0.02 M phosphate buffer, pH 7.0 were centrifuged (20,000 $\times g$, 10 min). The collected bacteria were suspended in phosphate buffer and washed by centrifugation two times. The bacteria were re-suspended in 0.155 M saline and applied to a glass wool column.¹¹⁾ The eluted suspensions were used for the tests. Pea seedling roots, which were grown aseptically in the growth pouch containing nutrient solution¹⁰⁾ with or without calcium nitrate (0.05 g/liter), were soaked in the above bacterial suspensions (pH 5.5~5.8, $4 \times 10^8 \sim 4 \times 10^{10}$ cells/ml) at 30°C for 2~16 hr with gentle shaking. The pea seedling roots were then washed in 0.155 M saline with stirring. The washed roots were cut off and root segments were set on a microscope slide, mounted in tap water and observed under a phase-contrast microscope (Olympus).

Fluorescence microscopy. Pea seedling roots soaked in the bacterial suspensions were washed and incubated at 30°C for 2 hr in PBS* (0.02 M, pH 7.0) containing 0.5~1 mg protein/ml immunoglobulin fraction of the appropriate anti-*Rhizobium* strain antibody. The seedling roots were washed with the buffer, re-incubated at 30°C in PBS containing 2 mg protein/ml FGAR (ICN Pharmaceuticals, Inc.) and re-washed. The root segments were set on a microscope slide, mounted in glycerol diluted ten times with PBS (0.05 M, pH 7.0) and then observed under a fluorescent microscope (Olympus) equipped with a HBO-100 lamp, using UG-1 exciter filter and L435 absorbing filter. As a control, pea seedling roots soaked in the saline alone were treated as described above and examined by the microscope under the same conditions.

Quantitative assay for adsorption of rhizobial cells to a pea root.

* Abbreviations; capsular polysaccharide, CPS; lipopolysaccharide, LPS; phosphate buffer-0.155 M saline, PBS; peak I, P-I; fraction, Fr.; fluorescein isothiocyanate, FITC; 2-keto-3-deoxyoctonate, KDO; FITC-labeled Ig G fraction of goat anti-rabbit gamma globulin, FGAR.

D-[U-¹⁴C] glucose (15~25 μ Ci) was added to the rhizobial culture (6~12 ml) in the late log phase, and incubated with shaking at 25°C for 2 hr. The ¹⁴C-labeled bacteria were re-suspended in 0.155 M saline as described above. Pea seedlings (after one to two days of germination), were selected that had roots of uniform shape and size, which showed similar numbers and distribution of root hairs on the surface. Four to eight seedling roots were immersed in the above bacterial suspension (4×10^8 cells/ml, 2 ml per two seedling roots). After incubation for 2~16 hr, the seedling roots were washed. To account for radioactivity taken up from solution rather than in the form of ¹⁴C-labeled bacterial cells, the ¹⁴C-labeled suspensions, in which pea seedling roots were not immersed, were incubated in the same way, and they were passed through 0.2 μ m membrane filters (Toyo Roshi Co., LTD). Pea seedling roots were incubated in the filtrate and treated in the same way as roots exposed to the bacterial suspension. One washed root, 3.2 cm in length, was divided into four equal parts, set on a piece of filter paper (1.5 $\times$ 1 cm) and dried. The radioactivity of the four root segments on the filter paper was measured in toluene (1 l)-PPO (1 g)-dimethyl POPOP (0.2 g) with a liquid scintillation counter (Aloka). The mean radioactive count of roots immersed in the filtrate was subtracted from the value of radioactivity of each root immersed in the bacterial suspension, and the difference, divided by the value of the radioactivity of bacterial cells (3~5 cpm/ 10^4 cells), gave the number of adsorbed cells per 3.2 cm root.

Preparation of heat-treated R. leguminosarum J357 cells.

¹⁴C-labeled or non-labeled bacteria were washed as described above and suspended in the phosphate buffer (ca. 10^6 cells/ml). The suspension was heated in a boiling water bath for 2 hr, cooled, and washed by centrifugation with the phosphate buffer. The saline suspension of the heat-treated bacteria was prepared as described above.

Effect of heat-treated bacteria, cell surface polysaccharide fractions or antibodies on adsorption of Rhizobium cells to a pea root.

Roots were immersed in the ¹⁴C-*Rhizobium* suspension in the presence of heat-treated bacteria, cell surface polysaccharide fractions or antibodies and then adsorbed cells per root were computed as described above.

Procedure for isolation and fractionation of rhizobial cell surface polysaccharides.¹⁰⁾

Saline extraction. Rhizobial cells harvested from liquid shaken culture by centrifugation were suspended in 0.155 M saline, stirred for 30 min at room temperature. This extraction was repeated twice. Afterwards,

the bacteria were washed with the saline by centrifugation. The combined supernatants were sterilized by passing through a membrane filter and dialyzed against water. The resulting solution was stored as a lyophilized preparation at -20°C .

Cetavlon precipitation and ethanol precipitation. An 0.5% aqueous solution of the above extract in 1.5% cetavlon solution was stirred for 10 min, and the precipitate and supernate fractions were separated by centrifugation. The precipitate fraction was dissolved in a small amount of 1.0 M saline, poured into ten volumes of ethanol and this mixture was stirred for 4 hr at 4°C . The precipitate which formed was collected by centrifugation, washed by ethanol, dissolved in water and dialyzed against water. The resulting solution was lyophilized. The supernate fraction was poured into ten volumes of ethanol, and processed as above.

Ion-exchange chromatography of crude LPS on DEAE-cellulose in Triton X-100. Crude LPS (500 mg) dissolved in 25 ml of a 1% Triton X-100 solution was applied to a column (3.5 $\times$ 20 cm) of DEAE-cellulose (DE 52, Whatman Ltd.) equilibrated with 1% Triton X-100. The column was washed with 400 ml of 1% Triton X-100 and eluted with a linear 0 to 0.5 M NaCl gradient. Total carbohydrate and KDO were measured in each non-hydrolyzed fraction and sugar-containing fractions were collected. Triton X-100 was extracted from the fractions with ethyl ether-ethanol according to D. E. Koeltzow *et al.*⁽²⁰⁾ Each aqueous fraction was concentrated under reduced pressure and poured into ten volumes of ethanol. The precipitated polysaccharides were dissolved in water and dialyzed. The aqueous solutions were stored as lyophilized preparations at -20°C .

Agarose gel electrophoresis of polysaccharides.

Polysaccharides (10 mg/ml) were subjected to agarose gel electrophoresis on a microscope slide (26 $\times$ 76 cm) layered with 2 ml of 0.85% agarose in 0.05 M barbital buffer, pH 8.5 at a current of 5 mA/slide. Polysaccharide was located as a precipitation band after fixing the slide in 95% ethanol at 4°C overnight.

Preparation of bacterial antigens.

Rhizobia were grown on agar medium consisting of 1 g of dipotassium hydrogen phosphate, 1 g of potassium dihydrogen phosphate, 0.6 g of potassium nitrate, 0.25 g of magnesium sulfate pentahydrate, 0.01 g of ferric chloride hexahydrate, 0.067 g of calcium chloride dihydrate, 25 μg of biotin, 100 μg of thiamine hydrochloride, 1 mg of boric acid, 1 mg of zinc sulfate heptahydrate, 0.5 mg of cupric sulfate pentahydrate, 0.01 mg of sodium molybdate, 10 g of mannitol and 15 g of agar per liter. Bacteria were harvested with sterile 0.02 M phosphate buffer (pH 7.0) and centrifuged. Cells were

suspended in the buffer and washed twice by centrifugation. Cells were re-suspended in sterile 0.155 M saline, and passed through a sterile glass wool column. Bacterial suspensions in saline (3×10^8 cells/ml) were prepared as antigens just before injections.

Preparation of antisera.

The antigen preparation (0.5–1 ml) was emulsified with an equal volume of Freund's complete adjuvant (Difco). The emulsion was injected into the large thigh muscle of the hind leg of Japanese white rabbits. After one month, rabbits were bled from the marginal vein of the ear, and the titer of the serum which was obtained was checked by the agglutinating test and immunodiffusion test. The rabbits were then given subcutaneous injections of 1 ml bacterial suspension in sterile saline (without adjuvant). Nine days after the second injection, blood was collected and the antiserum was isolated and stored at 4°C .

Isolation of immunoglobulin.

Fifty ml of PBS (0.02 M, pH 7.0) was added to 50 ml of immune or nonimmune serum and 100 ml of saturated ammonium sulfate was added to the PBS solution. The mixture was stirred at 4°C for 2 hr and the precipitate which formed was collected by centrifugation. The precipitate was dissolved in 50 ml of PBS, 33 ml of saturated ammonium sulfate was added and the mixture stirred at 4°C for 2 hr. The precipitate formed was collected, dissolved in PBS and dialyzed against PBS. Sodium azide was added to the resulting solution to make a 0.02% solution and the sample was stored at 4°C .

Immunochemical methods.

Gel diffusion. Gel diffusion plates were prepared with PBS (0.01 M, pH 7.0, 0.05% sodium azide) in 0.75% agar (Difco, Agar Noble). After diffusion, plates were washed with 0.155 M saline several times, followed by distilled water, dried at room temperature, and finally stained with Amido Black 10B.

Interfacial ring test. Antibody, pea seed lectin (1%) and nonimmune immunoglobulin solutions in PBS (0.02 M, pH 7.0) or the PBS alone were poured into separate microtubes (4 $\times$ 50 mm), and overlaid carefully with each solution of double dilution series of antigen (0.5%) in the PBS or the buffer solution. After overlaying, the occurrence of the precipitin band at the interface was observed during 5 hr at room temperature.

Agglutinating test. Equal volumes of antibody solution of double dilution series, buffer solution alone or nonimmune immunoglobulin were added to bacterial antigen (25 or 50 μl , $\text{OD}_{600}=0.5$). Agglutinations were observed at room temperature. The titer of the

antibody was determined as the maximum dilution multiple of the antibody solution with which agglutination was recognized.

Adsorption of bacterial cell surface polysaccharides to pea root hairs.

Two seedling roots, grown for 4 days aseptically, were soaked in 2 ml of bacterial polysaccharide fraction in PBS (0.5%) or PBS alone at 30°C for 2~6 hr, after which they were washed in 0.155 M saline for 10 min. They were tested by the indirect immunofluorescence technique using the appropriate anti-*Rhizobium* strain antibody and FGAR. Reactivities between antibodies raised against *Rhizobium* cells and polysaccharides were tested by gel diffusion and the interfacial ring test. All the titers of polysaccharide antigens (Fr. I-VI) by the interfacial ring test were more than 16.

Determination of uronic acid.

The amount of uronic acid in samples was determined by the carbazole-sulfuric acid method²¹⁾ using glucuronic acid as a standard.

Determination of heptose.

The amount of heptose in samples was determined by the Osborn method²²⁾ using glucoheptose as a

standard.

RESULTS

Adsorption of Rhizobium cells to pea root hairs

Phase-contrast microscopy. When pea roots were immersed in a suspension of *R. leguminosarum* J357 or J2080 ($4 \times 10^7 \sim 4 \times 10^9$ cells/ml), cells were observed to adsorb to the surface of pea root hairs in an end-on fashion¹²⁾ (Fig. 1a.). As the concentration of bacteria increased, bacterial cells were adsorbed to root hairs more densely. When a suspension containing less than 10^7 cells/ml was used, it was difficult to detect adsorbed cells. On the other hand, when suspensions contained more than 10^{10} cells/ml, masses of bacteria were observed between root hairs in places, and only few were adsorbed to the surface of the root hairs. Table I shows the results of adsorption of rhizobial strains (4×10^8 cells/ml), symbionts and non-symbionts of pea, to pea root hairs. Symbionts, *R. leguminosarum* J357 and J2080,

FIG. 1. Adsorption of *R. leguminosarum* J357 Cells (a) and Non-adsorption of *R. meliloti* 105 Cells (b) and Heat-treated J357 Cells (c) to Pea Root Hairs.

Pea seedling roots were incubated with the bacterial suspensions (4×10^8 cells/ml) for 16 hr at 30°C. The roots were observed under a phase microscope.

TABLE I. ADSORPTION OF *Rhizobium* CELLS TO *Pisum sativum* L. ROOT HAIRS

Species	Adsorption			
	Strain	Phase-contrast microscope	Fluorescent microscope	Nodulating host
<i>R. leguminosarum</i>	J357	+	+	Pea
	J2080	+		Pea
	J357-HT ^a	—		—
<i>R. meliloti</i>	105	—	—	Alfalfa
	J7017	—		Alfalfa
	J702	—		Alfalfa
<i>R. japonicum</i>	509	—		Soybean
	5033	—		Soybean
	501	—		Soybean
	J10	±		Soybean
<i>R. phaseoli</i>	7P	—		Kidney bean

^a HT, heat-treated in a boiling water bath for 2 hr.

adsorbed to pea root hairs (Fig. 1a) but non-symbionts, *R. meliloti* strains 105, J7017 and J702, *R. japonicum* strains 509, 5033 and 501 and *R. phaseoli* strain 7P did not adsorb to root hairs (Fig. 1b). *R. japonicum* strain J10 adsorbed to root hairs to only a small extent. Heat-treated *R. leguminosarum* J357 did not adsorb to root hairs (Fig. 1c). When pea roots were incubated with more than 10^{10} cells/ml of non-symbionts, masses of cells were observed between root hairs which was similar to the result obtained with the symbiont *R. leguminosarum*. Therefore, adsorption of bacteria to root hairs in an end-on fashion seems to be host-specific.

Fluorescence microscopy. Autofluorescence of pea root hairs was very weak (Fig. 2c), but that of the root base tissue was strong in the green-blue region. Fluorescence displayed by adsorption of anti-*R. leguminosarum* J357 (Fig. 2d), anti-*R. meliloti* 105 and FGAR was very weak on pea root hairs. Pea seedling roots immersed in *R. leguminosarum* J357 suspension were treated by the indirect immunofluorescence technique and fluorescent cells adsorbed to root hairs were observed (Fig. 2a). However, fluorescent cells were not observed in the root hair region of roots immersed in *R. meliloti* 105 (Fig. 2b). These results suggest that adsorption of *Rhizobia* to pea root hairs could be host-specific and the

adsorption site for specific binding to pea root hairs, exists on the symbiont cell surface.

Quantitative assay for adsorption of ¹⁴C-*Rhizobia* to a pea root

It was difficult to count cells adsorbed to pea root hairs directly.¹²⁾ Therefore, using ¹⁴C-labeled *Rhizobium*, the numbers of ¹⁴C-cells adsorbed to a pea root were measured indirectly. The radioactive counts of ¹⁴C-soluble materials, which might be ¹⁴C-glucose and metabolites released from the ¹⁴C-*Rhizobium*, was 13~20% of that of pea roots incubated with ¹⁴C-bacterial suspension. To obtain reproducible data, young homologous pea seedlings were selected, the incubation time was shortened (2 hr) to reduce non-specific adsorption of radioactive cells and materials to pea root, and a high concentration of bacterial suspension (more than 10^9 cells/ml) was not used (see METHODS). Under these conditions, the mean numbers±standard deviation of five replicates of ¹⁴C-*R. leguminosarum* J357 cells adsorbed to a pea root were 112 ± 19 , 121 ± 22 , 130 ± 18 , 124 ± 41 and 134 ± 12 , and reproducible data were obtained. There was no significant difference in the mean number of ¹⁴C-*R. leguminosarum* J357 cells adsorbed to a pea root between pH 4 and 7. Time course changes in the number of adsorbed cells are given in Fig. 3. The number of adsorbed cells reached the maximum in eight hours after the

FIG. 3. Time Course Changes of ¹⁴C-*R. leguminosarum* J357 Cells Adsorbed to a Pea Root.

Four to eight pea seedling roots were incubated with ¹⁴C-J357 cells (4×10^8 cells/ml) at 30°C. Points and bars show means and standard deviation.

incubation started.

The numbers of various ¹⁴C-*Rhizobium* strains adsorbed to a pea root in two hours are given in Table II. The value for the non-

TABLE II. NUMBER OF ¹⁴C-*Rhizobium* CELLS ADSORBED ($\times 10^{-4}$) TO A PEA ROOT OF 3.2 CM IN LENGTH

Species	Strain	Cells adsorbed to a root (means $\pm$ SD ^a)	Adsorption to root hairs ^b
<i>R. leguminosarum</i>	J357	134 $\pm$ 22	+
<i>R. leguminosarum</i>	J357-HT	120 $\pm$ 4	—
<i>R. meliloti</i>	105	105 $\pm$ 27	—
<i>R. japonicum</i>	509	43 $\pm$ 11 ^c	—

^a SD, standard deviation.

^b Examined by phase-contrast microscopy.

^c The value was significantly less than the mean number of *R. leguminosarum* J357 ($P \leq 0.001$).

symbiont ¹⁴C-*R. japonicum* 509 cells was significantly less ($P \leq 0.001$) than the mean number of the symbiont ¹⁴C-*R. leguminosarum* J357 cells. However, no significant difference in the mean number of adsorbed cells was observed among non-symbiont ¹⁴C-*R. meliloti* 105, heat-treated ¹⁴C-*R. leguminosarum* J357 and ¹⁴C-*R. leguminosarum* J357. ¹⁴C-*R. meliloti* 105 and heat-treated ¹⁴C-*R. leguminosarum* J357 could not adsorb to pea root hairs (Table II). Therefore, these cells might adsorb more non-specifically to root epidermal cells than

FIG. 4. Effect of Heat-treated *R. leguminosarum* J357 Cells on Adsorption of ¹⁴C-*Rhizobia* to a Pea Root.

Four pea seedling roots were incubated with ¹⁴C-*R. leguminosarum* J357 or ¹⁴C-*R. meliloti* 105 cells (4×10^8 cells/ml) in the presence of heat-treated J357 cells for 2 hr at 30°C. ○—○, *R. leguminosarum* J357; ×---×, *R. meliloti* 105.

root hairs and any significant difference might not be displayed. Moreover, this presumption is supported by the results in Fig. 4 which show the effect of heat-treated *R. leguminosarum* J357 cells at various concentrations on the adsorption of ¹⁴C-*R. leguminosarum* J357 or ¹⁴C-*R. meliloti* 105 to a pea root. As the concentration of heat-treated J357 cells increased, there was little change in the adsorption of ¹⁴C-J357 cells. On the contrary, adsorbed ¹⁴C-105 cells decreased to about 30% at 6×10^9 heat-treated cells/ml. Also, non-specific adsorption of ¹⁴C-105 cells was inhibited by the presence of the heat-treated cells. These results suggest that: (1) the adsorption site on a pea root of *R. meliloti* 105 is analogous to that of heat-treated *R. leguminosarum* J357 cells; (2) the adsorption site of *R. leguminosarum* J357 is different from that of *R. meliloti* 105 and heat-treated *R. leguminosarum* J357; (3) of the three bacterial cells, only *R. leguminosarum* J357 cells adsorb to pea root hairs significantly.

Isolation and fractionation of *Rhizobium* cell surface polysaccharides

It is supposed that rhizobial cell surface polysaccharides function in host-specific adsorption to root hairs. Cell surface polysac-

FIG. 5. Procedure for Isolation and Fractionation of Rhizobial Cell Surface Polysaccharides.

charides, containing CPS and LPS, were isolated and fractionated by the method as described previously¹⁰⁾ with some modification. The procedure is shown in Fig. 5. CPS was purified from the saline extract of *R. leguminosarum* J357, using the molar ratio of total carbohydrate to uronic acid as an indicator. The ratio of higher molecular P-I (Fr. II) obtained after gel filtration was approximately five times as great as that of the original saline extract. The P-I did not contain KDO and heptose, and gave one diffuse band by agarose gel electrophoresis.

Crude LPS obtained from the saline extracted J357, which co-aggregated with CPS, polysaccharides, nucleic acids and protein in aqueous solution to become a macromolecule, was dissolved in 1% Triton X-100, and applied to a DEAE-cellulose column equilibrated with 1% Triton X-100. The elution pattern is shown in Fig. 6. P-II contained mainly LPS, whereas P-III was more acidic and two times higher in the molar ratio of total carbohydrate to uronic acid than P-II. Precipitin bands of CPS purified from the saline extract and P-III were fused in the gel diffusion test. Therefore, P-III was regarded as the mixture of CPS or modified CPS, a trace of LPS and polysaccharides including polysaccharide degradation products of LPS. The higher molecular P-I (Fr. IV) by gel filtration was more purified LPS than the preparation obtained by the meth-

FIG. 6. Ion-exchange Chromatography of Crude LPS from *R. leguminosarum* J357 on DE-52 in Triton X-100.

Each fraction (10 ml) was assayed for total carbohydrate (○—○) at 490 nm and 2-keto-3-deoxyoctonate (×---×) at 549 nm.

od described previously.¹⁰⁾ This judgement is based on the two times higher molar ratio of total carbohydrate to KDO and three-fifth decreased ratio of total carbohydrate to uronic acid which was found.

Effect of cell surface polysaccharide fractions on adsorption of ¹⁴C-Rhizobia to a pea root

Using the polysaccharide fractions (Fr. I~VI) obtained from *R. leguminosarum* J357 or *R. meliloti* 105 (Fr. I~IV), effects of the polysaccharides on adsorption of ¹⁴C-rhizobia to a pea root were examined. CPS (Fr. II) from *R. leguminosarum* J357 inhibited the adsorption of ¹⁴C-J357 more strongly than CPS from *R. meliloti* 105 (Fig. 7). LPS (Fr. IV) from J357 strongly inhibited adsorption of ¹⁴C-J357, but LPS from 105 produced little effect on the adsorption (Fig. 8).

Table III summarizes the effect of each polysaccharide fraction from *R. leguminosarum* J357 as well as *R. meliloti* 105 on host-specific adsorption of ¹⁴C-J357 or non-specific adsorption of ¹⁴C-105 to a root. When pea roots were treated with Fr. II, Fr. IV or Fr. V of *R. leguminosarum* J357 polysaccharide fractions, the mean number of specifically adsorbed ¹⁴C-J357 cells was significantly less than the untreated control. However, non-specific adsorption of ¹⁴C-105 was not affected by those

FIG. 7. Effect of CPS on Adsorption of ^{14}C -*R. leguminosarum* J357 to a Pea Root.

Four to six pea seedling roots were incubated with ^{14}C -*R. leguminosarum* J357 cells (4×10^8 cells/ml) in the presence of CPS from *R. leguminosarum* J357 (○—○) or CPS from *R. meliloti* 105 (×---×) for 2 hr at 30°C.

FIG. 8. Effect of LPS on Adsorption of ^{14}C -*R. leguminosarum* J357 to a Pea Root.

Four to six pea seedling roots were incubated with ^{14}C -*R. leguminosarum* J357 cells (4×10^8 cells/ml) in the presence of LPS from *R. leguminosarum* J357 (○—○) or LPS from *R. meliloti* 105 (×---×) for 2 hr at 30°C.

fractions. When treated with Fr. I, II or III of *R. meliloti* 105 polysaccharide fractions, the mean number of non-specifically adsorbed ^{14}C -105 cells was significantly less than the untreated control, but Fr. I and Fr. III did not affect the specific adsorption of ^{14}C -J357 cells significantly.

The mean number of ^{14}C -*R. leguminosarum* J357 cells treated with 0.5% LPS from J357 adsorbed was significantly less ($P \leq 0.01$) than that of ^{14}C -J357 cells treated with 0.5% LPS from *R. meliloti* 105. Similarly, the number of

^{14}C -J357 cells treated with 1% CPS from J357 cells adsorbed was significantly less than when treated with 1% CPS from 105 cells. These results suggest that the specific adsorption site of *Rhizobium* to host root hairs exists in the rhizobial cell surface polysaccharides, CPS or LPS, and that CPS or LPS located on the cell surface of rhizobial cells have a specific structure related to the corresponding host recognition.

Adsorption of rhizobial cell surface polysaccharides to pea root hairs

To confirm the above hypothesis regarding the specific adsorption site, the capacity of adsorption of polysaccharide fractions to pea root hairs was examined by the indirect fluorescent antibody technique. Autofluorescence and fluorescence caused by binding of antibodies (Fig. 2c and 2d) on pea root hairs were very weak. CPS and LPS from the symbiont J357 bound to the surface of pea root hairs (Fig. 9a and 9c). However, CPS and LPS from the non-symbiont 105 did not bind to the surface of pea root hairs (Fig. 9b and 9d). Other polysaccharide fractions from both J357 and 105 could not bind to pea root hairs (Table IV).

Dazzo *et al.* reported that the fluorescence image made by binding of FITC-labeled capsular polysaccharide from *R. trifolii* to clover root hairs was strongest at the root hair tips and diminished towards the base of the root hairs.¹³⁾ In the case of pea root hairs, such gradient brightness in the fluorescence of pea root hairs could not be observed. This corresponded to the result that *R. leguminosarum* adsorbed to the whole root hair surface, although to some extent adsorbed more densely at the root hair tips than other root hair regions.

Antigenic analysis of rhizobial strains and their cell surface polysaccharides

Figure 10 shows that adsorption of ^{14}C -*R. leguminosarum* J357 cells in the presence of anti-J357 decreased to approximately 30% of that in the absence of the antibody, but when treated with anti-105 or non-immune immuno-

TABLE III. EFFECT OF POLYSACCHARIDES ON ADSORPTION OF *R. leguminosarum* J357 AND *R. meliloti* 105 TO A PEA ROOT

Treatment ^a	Adsorbed cells ($\times 10^{-4}$) ^b		Relative intensity	
	J357	105	J357	105
None	130 $\pm$ 18	106 $\pm$ 12	100	100
J357				
Fr. I	127 $\pm$ 5	155 $\pm$ 44	98	146
Fr. II (CPS)	53 $\pm$ 7 [†]	135 $\pm$ 69	41	127
Fr. II*	29 $\pm$ 6 [†]		22	
Fr. III	168 $\pm$ 6	114 $\pm$ 2	129	108
Fr. IV (LPS)	47 $\pm$ 4 [†]	121 $\pm$ 5	36	114
Fr. V	66 $\pm$ 7 [†]	91 $\pm$ 20	51	86
Fr. VI	139 $\pm$ 33	121 $\pm$ 5	107	114
105				
Fr. I	138 $\pm$ 32	10 $\pm$ 2 ^{††}	106	9
Fr. II (CPS)	70 $\pm$ 8 [†]	53 $\pm$ 3 ^{††}	54	50
Fr. II*	88 $\pm$ 21		68	
Fr. III	95 $\pm$ 12	41 $\pm$ 8 ^{††}	73	39
Fr. IV (LPS)	120 $\pm$ 26	119 $\pm$ 2	92	112

^a Concentration of polysaccharides noted by * was 1% and all the other polysaccharide concentrations were 0.5%.

^b Values were means $\pm$ standard deviation. Values noted by [†] and ^{††} were significantly less than respective untreated controls ($P \leq 0.01$).

FIG. 10. Effect of Antibodies on Adsorption of ¹⁴C-*R. leguminosarum* J357 to a Pea Root.

Four to six pea seedling roots were incubated with ¹⁴C-J357 cells (4×10^8 cells/ml) in the presence of nonimmune immunoglobulin (O—O), anti-J357 (×---×) or anti-*R. meliloti* 105 (Δ---Δ) for 2 hr at 30°C.

globulin, adsorption of ¹⁴C-J357 to a pea root was not affected. By the gel diffusion test, the antigenic difference between *R. leguminosarum* J357 and *R. meliloti* 105 was shown. J357 did not produce a precipitin band with anti-105, and 105 did not produce a precipitin band with anti-J357. Both J357 and 105 precipitin bands with the mixture of anti-J357 and anti-105

TABLE IV. ADSORPTION OF CELL SURFACE POLYSACCHARIDES TO PEA ROOT HAIRS

Species	Polysaccharide	Adsorption
<i>R. leguminosarum</i> J357	Fr. I	—
	Fr. II (CPS)	+
	Fr. III	—
	Fr. IV (LPS)	+
	Fr. V	+
	Fr. VI	—
<i>R. meliloti</i> 105	Fr. I	—
	Fr. II (CPS)	—
	Fr. III	—
	Fr. IV (LPS)	—

crossed without any interaction. These results imply that the two strains, *R. leguminosarum* J357 and *R. meliloti* 105, differ in the immunochemical structure of cell surface polysaccharides. Antigenic difference between CPS's from J357 and 105 is shown in Fig. 11. The two CPS's were non cross-reacting. Two LPS's obtained from J357 and 105, also did not cross-react. Therefore, it is expected that *R. leguminosarum* J357 has immunochemically different structures in both CPS and LPS from *R. meliloti* 105, and this corresponds to the different host recognition phenomena.

FIG. 11. Immunodiffusion Plates.

Each well was filled with 15~20 μ l of polysaccharides (0.1~0.5%) or antibodies (10~30 mg protein/ml). Center well, CPS from J357; Well 1, CPS from 105; Wells 2 and 3, anti-J357+anti-105; Well 4, CPS from 105; Well 5, anti-J357; Well 6, anti-105. Plates were incubated at 25°C in moist chambers for 24~48 hr.

Precipitin reaction between polysaccharide fraction and pea seed lectin

It is assumed that host-symbiont recognition is based on interaction between plant lectins and bacterial cell surface polysaccharides. Reaction titers of polysaccharide fractions from *R. leguminosarum* J357 as well as *R. meliloti* 105 were examined by the serial double dilution method (Table V). LPS and

TABLE V. PRECIPITIN REACTION BETWEEN POLYSACCHARIDE FRACTIONS AND PEA SEED LECTIN

Polysaccharide	Titer ^a of 0.5% polysaccharide	
	<i>R. leguminosarum</i>	<i>R. meliloti</i>
	J357	105
Fr. I	16	16
Fr. II (CPS)	64	—
Fr. III	—	NT ^b
Fr. IV (LPS)	4	—
Fr. V	16	NT
Fr. VI	—	NT

^a Measured by interfacial ring test (See the Methods).

^b NT, not tested.

CPS from symbiont J357 reacted with pea seed lectin, but LPS and CPS from non-symbiont 105 were non-reacting.

DISCUSSION

The initial infection process in host legume-*Rhizobium* associations may be one of the most important steps for the expression of host-specific root nodule formation. Especially taking the lectin hypothesis into consideration, it is expected that a *Rhizobium* recognizes the corresponding host in the adsorption process to bind specifically to host root hairs. However, there are few published reports on the qualitative and quantitative analysis of rhizobial adsorption to host roots. Dazzo *et al.* demonstrated that adsorption of *Rhizobium* to clover root hairs was well related to host specificity in *Rhizobium*-clover symbiosis.¹²⁾ This investigation was a study of whether adsorption of *Rhizobium* to pea root corresponded to host specificity in the *Rhizobium*-pea symbiosis. Previously, Chen *et al.* assayed adsorbed cells to pea roots using $1.5 \sim 3.0 \times 10^{10}$ cells per ml of ³²P-labeled *Rhizobia*.¹⁶⁾ It was our observation, that such a highly concentrated bacterial suspension could adsorb to the surface of pea root hairs only to a small extent and that mass formed by the bacteria was found between root hairs in places bearing no relation to host-symbiont associations. The qualitative and quantitative data given in this report show that the adsorption of *Rhizobium* to pea root hairs is related to host specificity. The ¹⁴C-labeled non-symbiont *R. japonicum* 509 cells bound to the roots in significantly less numbers than the symbiont *R. leguminosarum* J357 cells, but there was no significant difference in the number of adsorbed cells between live and heat-treated J357 and non-symbiont *R. meliloti* 105 (Table II). In this assay system, both root hairs and other epidermal root cells were exposed to the bacterial suspension. The heat-treated J357 or *R. meliloti* 105 did not bind to root hairs, but bound more to epidermal surface other than root hairs. However, heat-treated J357 cells competitively inhibited the adsorption of non-symbiont 105 cells but not the adsorption of symbiont J357 cells. It was supposed that the adsorption sites of non-symbiont 105 cells on pea root surface differed from that of symbiont

J357 cells. Also, the number of J357 cells adsorbed to a pea root in the presence of CPS or LPS from J357 was significantly less than that of 105 cells adsorbed to it under the same conditions. This may be due to the inhibition of adsorption of J357 cells to root hairs (observed by phase-contrast microscopy). Thus, the molecular structure on the bacterial cell surface related to adsorption of symbiont J357 cells to pea roots seemed to be different from those of non-symbiont 105 cells. Tables III and IV show that the cell surface polysaccharides, CPS or LPS, of *R. leguminosarum* J357 bind specifically with the receptor sites on pea root hairs to function as the host-specific adsorption of the *Rhizobium* to pea root hairs. CPS from *R. meliloti* 105 inhibited, more weakly than CPS from *R. leguminosarum* J357, the adsorption of J357 cells to a pea root. The results showing non-adsorption of 105 cells and CPS from 105 cells to pea root hairs, and inhibition of non-specific adsorption of 105 cells to a pea root by CPS from 105 cells, suggest that the CPS might inhibit non specific adsorption of J357 cells to other epidermal root cells. Also, the decrease in J357 cells adsorbed to pea roots was perhaps caused by non-specific, steric influence of CPS from 105 cells. In either case, chemical analysis of the adsorption sites is needed to elucidate this problem.

This report showed that: (1) adsorption of rhizobia to pea root hairs was host-specific; (2) cell surface polysaccharide, CPS or LPS from *R. leguminosarum* J357, specifically inhibited adsorption of the symbiont to a pea root; (3) the CPS or LPS was adsorbed specifically to pea root hairs; (4) the CPS or LPS reacted specifically with pea seed lectin. These results support the hypothetical model in which the host specificity in *Rhizobium*-pea symbiosis is determined by the interaction at the initial infection process between rhizobial cell surface polysaccharides and lectins which exist possibly on the surface of pea root hairs. Very recently, J. W. Kijne *et al.* reported that a glucose-binding protein with the same electrophoretic mobility as the one of seed lectins was isolated from pea root slime.²³⁾ In our preli-

minary experiments, the pea seed lectin-receptor on pea root hairs was indicated by the fluorescent antibody technique. Also, substances which reacted specifically with the antibody against pea seed lectin were found in the sterile nutrient solution in which the pea seedling roots were immersed. These results suggest that lectins cross-bridge the polysaccharides on the cell surface of *R. leguminosarum* and pea root hairs in a manner as proposed by the model of Dazzo *et al.*¹¹⁾ Both CPS and LPS from *R. leguminosarum* J357 had (a) inhibitory activity on the adsorption of J357 cells to a pea root, (b) the capacity to adsorb to pea root hairs, and (c) precipitin reactivity with pea seed lectins, so that, it is inferred that there are analogous reaction sites to the receptor on pea root hairs in both polysaccharides. However, the respective roles of the CPS and the LPS in the infection step remain to be resolved.¹⁹⁾ It is anticipated that CPS of encapsulated bacteria or LPS of bacteria which are decapsulated in rhizosphere conditions bind with the receptor on root hairs in the initial meeting, and that interaction between LPS and the receptor of the root hair surface trigger the next steps, root hair penetration and infection thread formation after the adsorption of CPS to root hairs.

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FIG. 2. Fluorescence Microscopy of Pea Root Hairs: (a) Fluorescence of *R. leguminosarum* J357 Cells Adsorbed to Pea Root Hairs; (b) Pea Root Hairs Incubated with *R. meliloti* 105 Cells; (c) Untreated Pea Root Hairs (Autofluorescence); (d) Pea Root Hairs Incubated with PBS before Treatment of Indirect Immunofluorescent Antibody Technique Using Anti-J357 and FGAR (See METHODS).

The backgrounds are autofluorescence of pea root tissues.

FIG. 9. Immunofluorescence of Pea Root Hairs Incubated with Bacterial Cell Surface Polysaccharides.

Pea root hairs were incubated with (a) 0.5% CPS from *R. leguminosarum* J357, (b) 0.5% CPS from *R. meliloti* 105, (c) 0.5% LPS from J357 and (d) 0.5% LPS from 105. Incubation was carried out for 6 hr at 30°C.

FIG. 2.