

Carbohydrate Binding Activities of *Bradyrhizobium japonicum*.

I. Saccharide-specific Inhibition of Homotypic and Heterotypic Adhesion

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Abstract. *Bradyrhizobium japonicum* (R110d) exhibited four saccharide-specific binding activities: (a) adsorption to Sepharose beads containing covalently coupled lactose; (b) homotypic agglutination through one pole of the cell (star formation); (c) heterotypic adhesion to the cultured soybean cell line, SB-1; and (d) attachment to roots of soybean plants. Each of these binding activities can be inhibited by the addition of galactose or lactose, but not by derivatives such as *N*-acetyl-D-galactosamine or melibiose. Treatment of wild-type bacteria with *N*-methyl-*N'*-nitro-*N*-

nitrosoguanidine followed by selection on the basis of reduced binding to SB-1 cells, resulted in two specific mutants, designated N4 and N6. Compared to wild type, these two mutants also exhibited decreased binding activity in: (a) adsorption to lactose-Sepharose beads; (b) homotypic star formation; and (c) heterotypic attachment to roots of soybeans plants. These results suggest that all four of the saccharide-inhibitable binding activities of *Bradyrhizobium japonicum* may be mediated by the same mechanism(s) or molecular component(s).

LEADING to the formation of nodules and a nitrogen-fixing symbiosis, *Bradyrhizobium japonicum* normally infects the roots of soybean plants (1). In previous studies, we had reported the binding of *B. japonicum* to cells of the cultured soybean line, SB-1, originally derived from the roots of *Glycine max* (12, 13). This binding interaction appeared to be mediated via carbohydrate recognition, since galactose (Gal)¹ can inhibit the heterotypic adhesion, but glucose cannot. Although there was evidence for a galactose-specific lectin, immunologically cross-reactive with soybean agglutinin (SBA), on the cell wall or outer surface of SB-1 cells (12, 20), there was less than definitive evidence that this plant lectin can account for the saccharide specificity of the *Rhizobium*-soybean cell interaction. The most glaring discrepancy between the notion of plant lectin mediating the carbohydrate-specific recognition of bacteria and the experimental data was that while purified SB-1 cell lectin bound both Gal and *N*-acetyl-D-galactosamine (GalNAc) (19), the latter saccharide failed to inhibit *Rhizobium* binding to the SB-1 cells (12, 13). These results suggested that the saccharide specificity in the inhibition of *Rhizobium*-SB-1 cell binding may reflect recognition components, which exhibit exquisite Gal-specific characteristics, other than the plant lectin.

In the course of studies to analyze this specificity, we observed that *B. japonicum* can bind to beads covalently derivatized with lactose (Lac) in a carbohydrate-specific fashion. Moreover, several other binding activities of the intact bacte-

ria, including autoagglutination (star formation), adhesion to cultured soybean (SB-1) cells, as well as adhesion to roots of soybean plants, shared the same carbohydrate-binding specificity. We now document these observations in the present series of papers. In this communication, we report the carbohydrate-specific binding properties of *B. japonicum*. The accompanying paper describes the isolation and characterization of the galactose-specific *B. japonicum* lectin, BJ38 (14).

Materials and Methods

Bacterial Strains and Mutant Isolation

B. japonicum R110d was obtained from the late Dr. Barry Chelm (Michigan State University) and was cultured on yeast extract-mannitol-sodium gluconate (YEMG) medium as described before (12). *B. japonicum* R110d was mutagenized by *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) as described by Maier et al. (18). The bacteria were grown to exponential phase and 5 ml of the bacterial suspension was treated with 0.16 mg/ml MNNG in 50 mM Tris-maleate buffer (pH 6.5). The suspension was incubated at 30°C for 1 h and then centrifuged. The pellet was washed twice with 10 ml of YEMG. The mutagenized cells were suspended in 50 μ l YEMG and plated on YEMG agar slates (supplemented with 30 μ g/ml chloramphenicol) to allow growth of the survivors. After 5 d, bacteria were transferred to 50 ml of liquid medium (YEMG) and grown for 2 d. Bacterial suspension (5 ml) was then passed to another 50 ml of medium. After 16 h of growth, 0.5 ml of the bacterial culture was added to 20 ml of SB-1 cells (20% [vol/vol]). The mixture was incubated at 26°C for 8 h to allow attachment of bacteria to SB-1 cells. The mixture was then centrifuged at 800 *g* for 2 min to remove the SB-1 cells, together with the attached bacteria. The nonattached bacteria were collected by centrifugation at 8,000 *g* for 10 min and subcultured in YEMG medium. After 16 h of growth, the bacteria were subjected to four more rounds of incubation with the SB-1 cells followed by removal of bacteria bound to the soybean cells.

1. Abbreviations used in this paper: Gal, galactose; Lac, lactose; LPS, lipopolysaccharide; MNNG, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine; SBA, soybean agglutinin.

After five cycles of the absorption process, the nonbinding bacteria were diluted and plated on the solid YEMG agar medium supplemented with 30 $\mu\text{g/ml}$ chloramphenicol to generate colonies from single cells. 10 d after plating, each colony was individually tested for SB-1 cell binding. Cells were transferred from individual colonies by sterile toothpicks to separate wells containing 100 μl YEMG medium. After 16 h of growth at 26°C, 50 μl of the bacterial suspension was transferred to another well containing 50 μl of SB-1 cells (20% [vol/vol]). The mixtures were then incubated for 16 h at 26°C and then were examined under the microscope for SB-1 cell binding. About 0.5% of the bacterial colonies did not show any binding to the SB-1 cells. The colonies from the nonbinding bacteria were further tested to ascertain the nonbinding phenotype. Two clones were chosen for this study. The effect of MNNG on these clones is not known. However, these clones showed phenotypic differences in their binding ability to SB-1 cells as compared to the wild type. We designated these clones as mutants N4 and N6.

Immunological Cross-reactivity of Various Bacterial Strains with Anti-*B. japonicum* Antibody

The anti-*B. japonicum* antibody (anti-Brj) used in this study was characterized and reported previously (13). This antibody fraction contains mainly antibodies directed against the *B. japonicum* lipopolysaccharide (LPS). Besides the antibodies against LPS, this fraction also reacts with five other bacterial components as determined by immunoblotting analysis (13). Anti-Brj antibody was iodinated by the chloramine T method described by Langone (17).

The immunoreactivity of various bacterial strains with anti-Brj antibody was examined by incubating the different strains of bacteria with anti-Brj antibody (60 $\mu\text{g/ml}$ anti-Brj containing 10^5 cpm ^{125}I -anti-Brj) at room temperature for 3 h. Each sample was filtered through a 0.22- μm filter (Millipore Continental Water Systems, Naperville, IL) that had been presoaked in PBS (10 mM sodium phosphate, 0.14 M NaCl, 4 mM KCl, pH 7.4) containing 5% BSA to eliminate nonspecific binding. The membrane was washed three times with 2 ml PBS-BSA. Radioactivity that was retained on the filter was then determined.

LPS was purified from both the parental and mutant strains of *B. japonicum* as previously described (13). Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate was carried out by the procedure of Laemmli (16) with 10 and 4% (wt/vol) acrylamide in the running and stacking gel, respectively. After electrophoresis, the samples were electrotransferred to nitrocellulose membrane (400 mA for 2 h at 25°C). The membrane was treated with 5% BSA for 1 h and then incubated with 20 $\mu\text{g/ml}$ anti-Brj antibody for 3 h at room temperature. After washing, the membrane was incubated with goat anti-rabbit immunoglobulin conjugated with alkaline phosphatase (1:1,000 dilution; Sigma Chemical Co., St. Louis, MO). The immunoreactive components were revealed by alkaline phosphatase staining with 5-bromo-4-chloro-3-indonyl phosphate and nitro blue tetrazolium chloride as substrate.

Binding of *B. japonicum* to SB-1 Cells

The assay for rhizobium binding to SB-1 cells has been reported previously (13). SB-1 cells (0.5 ml of cells suspension, 20% [vol/vol], $\sim 10^6$ cells) of a 2-d-old culture were added to a 35-mm culture dish containing 1 ml of IB5C medium and then 0.5 ml of *B. japonicum* of appropriate concentration was inoculated into the dish. The mixtures were incubated for 16 h at 26°C without shaking. After incubation, cells were transferred to culture tubes and washed three times with 2 ml of PBS-BSA to remove unbound bacteria. The relative amounts of bacteria bound were compared by radioimmunoassay. The cells were incubated with 1 ml of anti-Brj (60 μg antibody/ml in PBS-BSA with 10^5 cpm ^{125}I -anti-Brj immunoglobulin). After incubation at room temperature for 3 h with shaking, the cells were washed three times with PBS-BSA. The amount of radioactivity was then determined. All experiments were done in triplicate. In parallel assays, bacteria were added to SB-1 cell samples at 0 time and harvested immediately thereafter to assess for background. All data were corrected for background accordingly. The amount of *B. japonicum* R110d (wild type) associated with the SB-1 cells at the highest dosage (1.0×10^9 cells/ml) was taken as 100%. The rest of the data were calculated as percentages relative to this value.

In this (SB-1 cell binding) assay, as well as the other heterotypic binding assays (binding to soybean roots and binding to beads containing covalently coupled saccharides), the conclusions derived from the quantitative assay using ^{125}I -labeled anti-Brj were confirmed qualitatively by microscopic examination. In all of the binding assays, including homotypic star formation,

the effects of saccharides were analyzed by carrying out the binding protocol in the presence of 50 mM saccharide. Where applicable, both quantitative radioimmunoassay and qualitative microscopic observation were performed.

Binding to *B. japonicum* to Soybean Roots

Soybean seeds were germinated at 26°C in the dark by wrapping them inside the sterile paper towels moistened with sterile distilled water. After 4 d, segments of the roots, ~ 1 -cm long, were excised aseptically from the root hair zone. These segments were divided into groups of 10 in 35-mm culture dishes and rinsed once with 2 ml of IB5C medium. The samples were treated with 2 ml of bacterial suspension at various doses as stated in the text. After incubation for 16 h at 26°C without shaking, the root segments were washed six times with 2 ml PBS-BSA in a rotary shaker for 10 min each to remove unbound bacteria. The samples were then assayed for bound bacteria by radioimmunoassay with anti-Brj antibody as described above for SB-1 cell binding. The data were expressed as relative percentages with the amount of wild-type bacteria associated with the root segments at the highest dosage (1.0×10^9 cells/ml) taken as 100%.

Star Formation Assay

The ability of various *B. japonicum* strains to achieve star formation (11, 26) was evaluated by culturing the bacteria on solid agar medium (YEMG) at 30°C for 10 d. The bacteria were picked up by a sterile toothpick and smeared on a microscope slide with a drop of water. The samples were examined under a phase-contrast microscope. Clusters of 10–20 cells joined at their tips were taken as positive for star formation. Under the assay conditions, $\sim 80\%$ of the cells from wild type showed stars, while the mutants demonstrated $<1\%$ star formation.

Binding of *B. japonicum* to Saccharide-containing Beads

The disaccharides Lac, maltose, and sucrose were coupled to aminoethyl polyacrylamide beads (aminoethyl Bio-Gel P100; Bio-Rad Laboratories, Richmond, CA) using sodium cyanoborohydride method of Baues and Gray (2). The reaction mixtures were agitated at room temperature for 4 d. The beads were washed and excess amino groups were blocked by N-acetylation by addition of acetic anhydride. The beads were then washed with water, 0.1 N NaOH, water, and PBS.

The coupling of Lac to Sepharose 4B beads was carried out using Matsumoto's procedure (21). Sepharose 4B (Pharmacia Fine Chemicals, Piscataway, NJ) was activated with epichlorohydrin and the resulting epoxy-activated beads were aminated by reaction with ammonia. After washing, the amino-Sepharose 4B beads were coupled with Lac for 4 d in the presence of sodium cyanoborohydride by reductive amination. Excess amino groups were then blocked by N-acetylation with acetic anhydride.

For assaying the binding of *B. japonicum* to saccharide-containing beads, the beads were first suspended in water (50% [vol/vol]). An aliquot (100 μl) was transferred to a 35-mm culture dish, with the addition of 0.9 ml of bacterial suspension (2×10^8 cells/ml) and 1 ml of water. To test for saccharide inhibition of the binding, this 1 ml of water was replaced with 1 ml of saccharide solution (100 mM). The samples were incubated for 16 h at 26°C without shaking. The samples were then transferred to polystyrene tubes (12 \times 75 mm) and washed to remove unbound bacteria with 2 ml of PBS-BSA by centrifugation (460 g for 1 min) and resuspension. The amount of bacteria bound to the beads was determined by microscopic observation or by radioimmunoassay using ^{125}I -anti-Brj immunoglobulin (13).

Results

Saccharide-specific Binding Activities of *Bradyrhizobium japonicum*

When cultured SB-1 cells were mixed with *B. japonicum* (R110d) at 26°C for several hours, washed, and sampled under a microscope, the bacteria adhered to soybean cells. A striking "polar" mode of binding was observed; the *Rhizobium* adhered to the plant cells in an "end-to-end" fashion. We have documented this specific interaction in previous

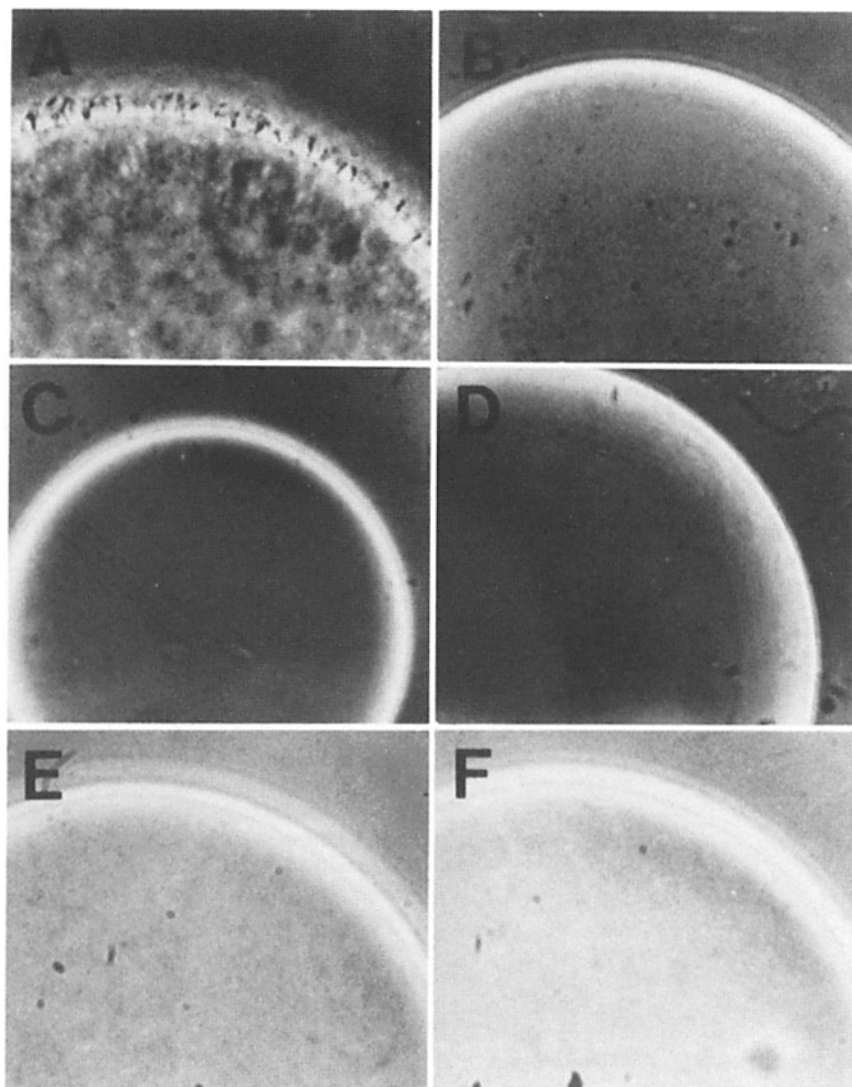


Figure 1. Binding of *B. japonicum* cells to polyacrylamide beads containing covalently coupled saccharides. Bacterial cells (1×10^8 cells/ml) were incubated for 16 h at 26°C with polyacrylamide beads derivatized with lactose (A), maltose (C), and sucrose (D). In B, the polyacrylamide beads were subjected to the derivatization procedure, but without the addition of any saccharides. In E and F, mutants N4 and N6, respectively, were incubated with Lac-Sepharose beads under the same conditions.

communications and have developed a quantitative assay for the binding of the bacteria to the soybean cells (12, 13). By using this assay, we showed that the recognition between *Rhizobium* and SB-1 cells was saccharide specific. The inclusion of Gal or Lac during the co-culture inhibited the binding of the bacteria to the soybean cells. In contrast, glucose, the C-4 epimer of Gal, and melibiose, a disaccharide isomer of Lac, did not show inhibition. Moreover, GalNAc, a derivative of Gal at the C-2 position, was also not inhibitory. These results indicate that the adhesion of *Rhizobium* to SB-1 cells may be mediated via a highly specific carbohydrate recognition system.

To test whether the lectin resided on the bacterial cell surface, we tested for the binding of *B. japonicum* to polyacrylamide beads covalently derivatized with various saccharides. We found that *B. japonicum* bound to polyacrylamide beads derivatized with Lac (Fig. 1 A). The bacteria did not bind to underivatized beads or to beads derivatized with maltose or sucrose (Fig. 1, B-D). Similarly, the *Rhizobium* bound to Lac-Sepharose beads (data not shown). The binding of the *Rhizobium* to Lac-Sepharose was inhibitable by 50 mM Lac and Gal, but not by the same concentrations of glucose and melibiose. GalNAc also failed to inhibit the binding.

By using the quantitative assay established for assessing the binding of *B. japonicum* to SB-1 cells, we have carried out a survey of the inhibitory capacity of various saccharides to block the adhesion of *Rhizobium* to Lac-Sepharose beads

Table I. Effect of Saccharides on *Rhizobium* Binding to Lac-Sepharose Beads*

Saccharide	Bound	Binding
	cpm	%
No hapten	5,623 ± 75	100
Glucose	4,910 ± 102	87.3 ± 1.8
Arabinose	4,986 ± 111	88.6 ± 1.9
Galactose	4,264 ± 251	75.8 ± 4.4
N-Acetyl-D-galactosamine	5,242 ± 129	93.2 ± 2.3
Lactose	1,984 ± 262	35.2 ± 4.6
Melibiose	4,805 ± 104	85.4 ± 1.8

* The binding assay was performed by incubating *B. japonicum* cells with Lac-Sepharose beads for 16 h at 26°C. The amount of bacteria associated with the beads was quantitated by radioimmunoassay with anti-Brj antibody as described in Materials and Methods. The final concentration of saccharide used was 10 mM. The data represent the means of triplicate determinations ± standard deviation.

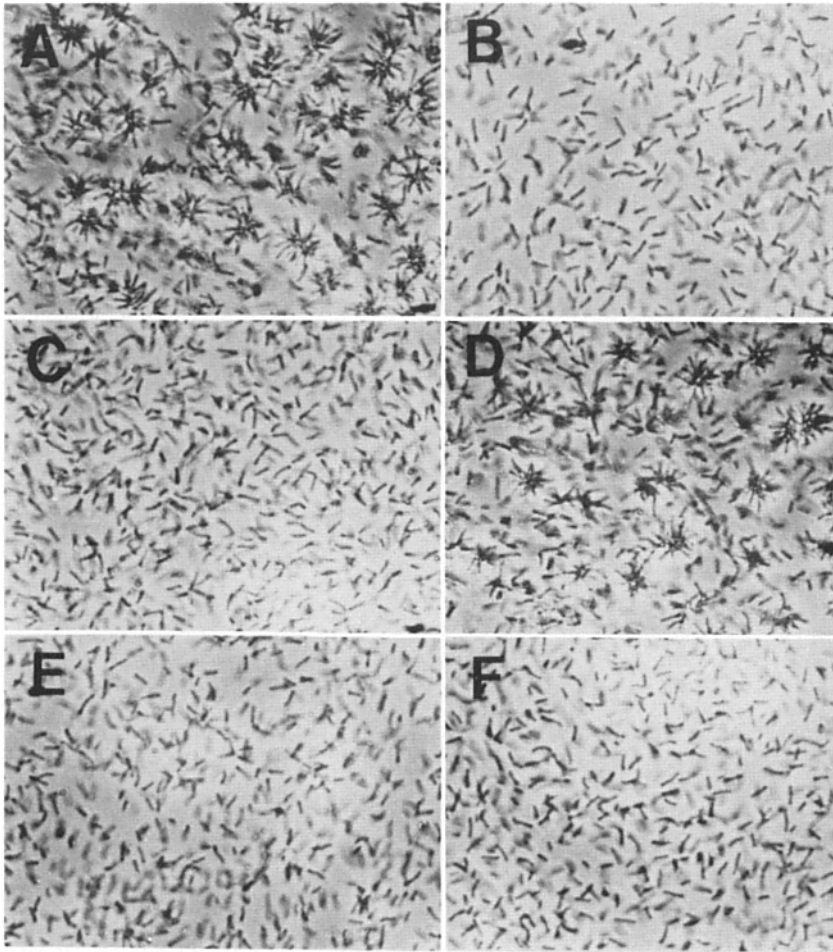


Figure 2. Star formation by *B. japonicum* and the effect of various saccharides. *B. japonicum* cells were cultured in solid agar YEMG medium in the absence or presence of saccharides at a final concentration of 50 mM. The bacteria were cultured for 10 d before observation under the microscope. Wild-type *B. japonicum* (R110d) was assayed in A–D. (A) Control; (B) lactose; (C) galactose; (D) *N*-acetyl-D-galactosamine. Mutants N4 (E) and N6 (F) were tested in the same assay.

(Table I). A slightly lower concentration of saccharides (10 mM) was chosen in order to distinguish variations in the relative affinities of the sugars. The numbers of bacteria bound to the beads in the presence of saccharides were compared by quantitating the ^{125}I -anti-Brj immunoglobulin. Lac was the most potent inhibitor of the binding of *Rhizobium* to Lac-Sepharose beads. While Gal also inhibited the binding, GalNAc and melibiose showed little or no inhibition. It should be noted that we had already demonstrated that at the concentrations used, the various saccharides had no effect on the binding of anti-Brj to the bacteria (13) during our quantitation assay. Therefore, the effect of saccharides is most reasonably ascribed to an effect on the binding of *B. japonicum* to the Lac-Sepharose beads. These results confirm the notion that the bacterial binding to Lac-Sepharose beads was mediated by a recognition protein that can exquisitely distinguish Gal from a derivative at the C-2 position, GalNAc.

Effect of Saccharides on Homotypic and Heterotypic Adhesion of *Bradyrhizobium japonicum*

When *B. japonicum* R110d cells were cultured in semisolid agar medium for 10 d, the bacterial cells joined together in a tip-wise orientation, forming starlike rosettes of 10–20 cells (Fig. 2 A). This corresponds to the ability of many rhizobia to aggregate through the interaction of one of the poles of the cell (autoagglutination or star formation) as has been previously documented for *R. phaseoli*, *R. lupini*, as

well as *B. japonicum* (4, 11, 26). In the course of the present studies on the effect of saccharides on the binding activities of *B. japonicum*, we observed that the addition of saccharides such as Lac also inhibited autoagglutination (Fig. 2 B). The specificity of saccharide inhibition again showed that while Gal blocked agglutination, GalNAc failed to yield the same effect (Fig. 2, C and D).

Since the cultured SB-1 cell line was originally derived from soybean roots, it was of interest to test whether saccharides also affected the binding of *B. japonicum* adhesion to root cells in vivo. Similar to SB-1 cell binding, *B. japonicum* bound to the soybean root in a saccharide-specific fashion. Under microscopic observation, the bacteria showed polar attachment to the soybean roots, while the presence of Lac and Gal blocked such binding (Fig. 3, A–C). GalNAc failed to inhibit this binding (Fig. 3 D).

All of these observations indicate that *Bradyrhizobium japonicum* exhibit four saccharide-specific binding activities: (a) adsorption to Lac-Sepharose; (b) homotypic autoagglutination; (c) heterotypic binding to SB-1 cells; and (d) heterotypic adhesion to soybean roots. The fact that the specificities of carbohydrate inhibition of all four assays were the same suggests that these processes may be mediated by the same component(s) and mechanism(s). One likely candidate responsible for mediating such saccharide-specific binding is the lectin, designated BJ38, that we have purified from *Bradyrhizobium japonicum* (14). Such a notion leads to

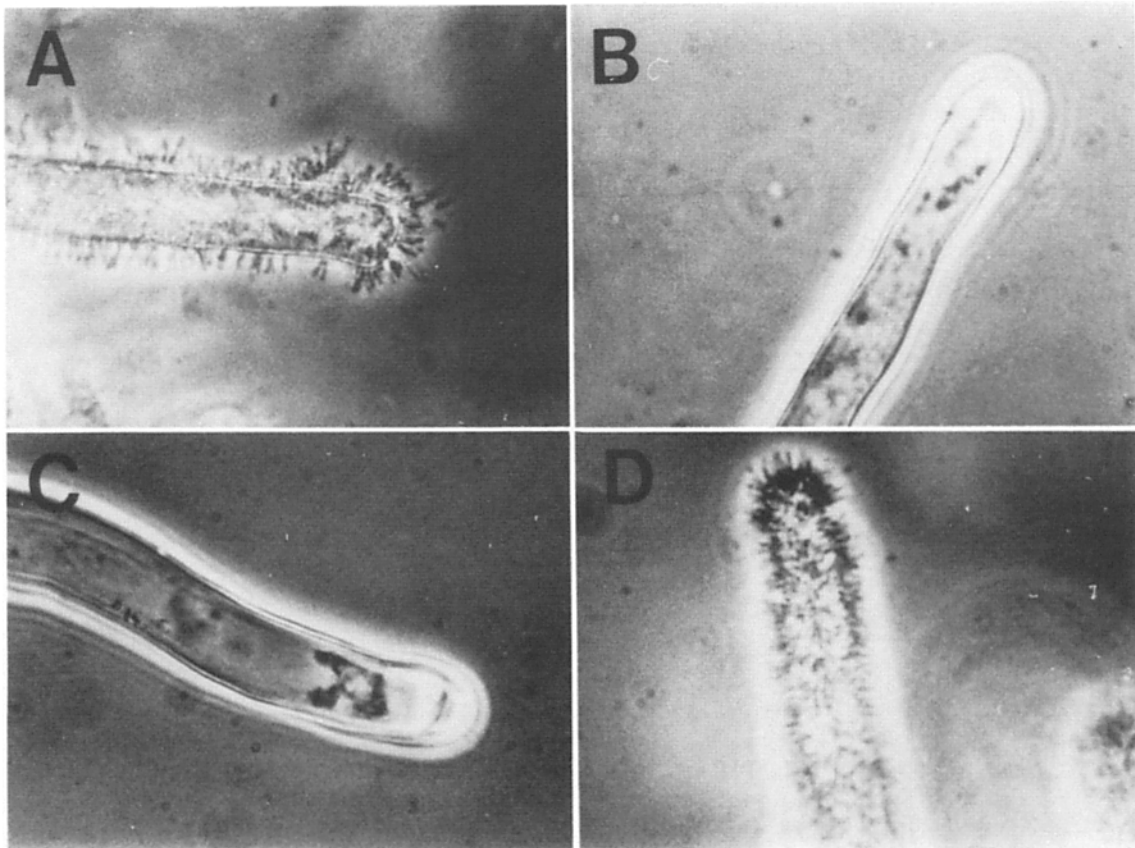


Figure 3. *B. japonicum* binding to soybean roots and the effect of saccharides. Soybean seeds were sterilized and germinated in the dark. The root hair zones were dissected aseptically and incubated with *B. japonicum* in the presence or absence of 50 mM saccharide. After 16 h of incubation at 26°C, the root segments were washed with PBS and then observed under the microscope. (A) Control; (B) lactose; (C) galactose; (D) *N*-acetyl-D-galactosamine.

two clear-cut predictions. First, isolation of mutants of *B. japonicum* based on impairment of one binding activity (e.g., SB-1 cell binding) should result in the concomitant loss of the other three binding activities (soybean root attachment, star formation, and binding to Lac-Sepharose) in the same mutant cells. Second, these mutants may produce little or defective molecules corresponding to BJ38.

Mutants of *B. japonicum* Defective in Binding to SB-1 Cells

Two *B. japonicum* mutants were obtained by MNNG mutagenesis and selection for their decreased ability (relative to wild type) to bind to cultured soybean cells. The binding of the wild type and the mutants to the SB-1 cells was quantitatively compared by radioimmunoassay (13). After the bacteria were co-cultured with the SB-1 cells for 16 h, the number of bacteria attached to the SB-1 cells was quantitated by ¹²⁵I-anti-Brj immunoglobulin. The mutants N4 and N6 were significantly decreased in SB-1 cell binding as compared to the wild type (Fig. 4).

Chemical mutation of bacteria may result in changes in bacterial surface components, thus possibly affecting our radioimmunoassay, which depends on the reaction of the antibody with the bacterial surface antigens. Therefore, two lines of experiments were performed to compare the reactivity of anti-Brj with the bacterial surface antigens of the wild-type

and mutant bacteria. First, we had shown previously that LPS of *B. japonicum* was one of the antigens recognized by our rabbit anti-Brj (13). When purified LPS of the mutants and wild-type cells were compared by immunoblotting with anti-Brj antibody, they all showed the same ladder patterns of immunoreactive bands (Fig. 5, A–C). Second, the binding of ¹²⁵I-labeled anti-Brj to the three bacterial cell types was quantitated; the wild type and the mutants showed the same level of binding of anti-Brj antibody (Fig. 5 D_j).

These results provide evidence that the MNNG mutagenesis did not affect the number of antigenic binding sites on the bacterial surface of the mutants. More importantly, these results also indicated that the decrease in bacterial binding of the mutants to the SB-1 cells, as detected by the radioimmunoassay, reflected the loss of binding ability of the mutants to the SB-1 cells, rather than a decrease in antibody cross-reactivity due to the loss of the bacterial surface antigens. Parallel microscopic observation provided confirmation of this notion; the mutants showed almost no binding to the SB-1 cells. Even after 2 d of incubation, these mutants did not show any increase in their binding to SB-1 cells.

The N4 and N6 mutants did not show any difference, from parental cells, in their growth rates in YEMG medium, as well as in low nutrient medium. Attempts to enhance their binding by manipulating their growth conditions, such as using bacteria of various growth phases or cultures in differ-

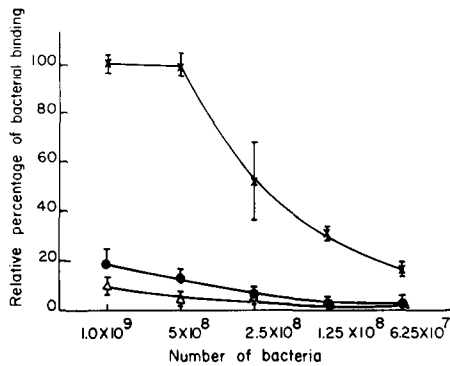


Figure 4. Comparison of SB-1 cell binding by *B. japonicum* R110d and its mutants. SB-1 cells were cocultured with various doses of bacteria. After 16 h of incubation at 26°C, the SB-1 cells were washed and the bound bacteria were determined by radioimmunoassay. (x) *B. japonicum* R110d; (●) N4; (Δ) N6.

ent growth media, were all unsuccessful. Finally, the general morphology and motility of the mutants, assayed by microscopic observation, did not appear to be affected. Thus, it seemed unlikely that MNNG mutagenesis caused a drastic change in bacterial metabolism, in general, leading to alteration of bacterial growth and, in turn, to changes in the binding properties of the mutant bacterial cells to SB-1 cultures.

Comparisons of the Other Saccharide-specific Binding Activities of Parental and Mutant Bacteria

The ability of the parental strain of bacteria, and its mutants, to attach to soybean roots was also examined by the radioim-

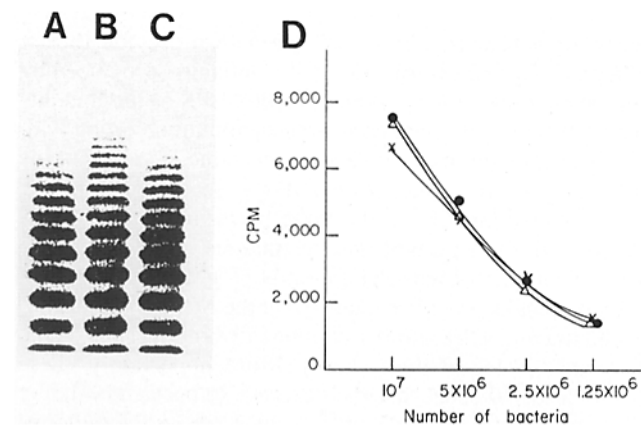


Figure 5. Comparison of the reactivity of anti-Brj with *B. japonicum* (R110d) and its N4 and N6 mutant cells. Immunoblotting analysis of LPS isolated from wild type and its mutants. Purified LPS was subjected to SDS-PAGE and immunoblotting analysis was performed with anti-Brj immunoglobulin as primary antibody and with alkaline phosphatase-conjugated goat anti-rabbit IgG antibody as secondary antibody. Lane A, LPS from *B. japonicum* R110d; lane B, LPS from N4; and lane C, LPS from N6. (D) Quantitation of amount of ^{125}I -labeled anti-Brj bound to wild-type and mutant cells. The bacteria were incubated with anti-Brj antibody (60 $\mu\text{g}/\text{ml}$ with 10^5 cpm ^{125}I -anti-Brj antibody). After 3 h of incubation, the amount of radioactivity associated with the bacteria was determined by filter assay. (x) *B. japonicum* R110d; (●) N4; (Δ) N6.

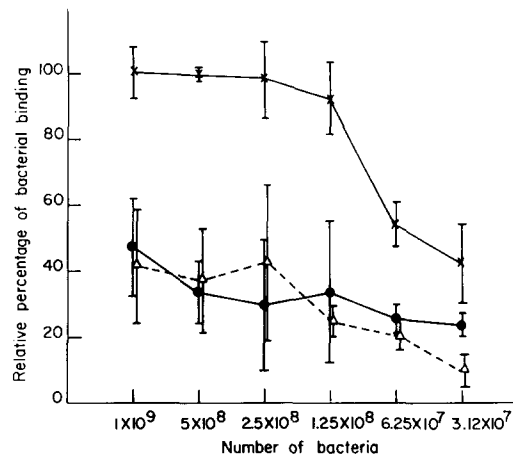


Figure 6. Comparison of soybean root binding by *B. japonicum* R110d and its mutants. Soybean root segments were incubated with various doses of the bacteria for 16 h at 26°C. After incubation, the roots were washed extensively and the bound bacteria were quantitated by radioimmunoassay. (x) *B. japonicum* R110d; (●) N4; (Δ) N6.

unoassay. Similar to SB-1 cell binding, *B. japonicum* R110d cells bind to the soybean root in a dose-dependent fashion, while mutants N4 and N6 showed a significant decrease in soybean root attachment (Fig. 6). When observed under the microscope, wild-type *B. japonicum* R110d exhibited firm and polar attachment to the soybean roots. On the other hand, both mutants, even at the highest dosage tested, failed to show any polar attachment to the roots. Occasionally, some mutant bacteria remained trapped or loosely associated with the roots, which may explain the residual binding activity as detected by the radioimmunoassay.

The binding of wild-type R110d cells and the N4 and N6 mutants to Lac-Sepharose beads was also compared qualitatively by microscopic observation (Fig. 1, E and F). There was a drastic reduction in the binding to the saccharide-containing beads by the mutants N4 and N6, as compared to the parental bacteria.

Finally, the autoagglutination assay was carried out in semisolid agar medium. While star formation was observed in cultures of wild-type *B. japonicum* (Fig. 2 A), no such polar aggregation was found in parallel cultures of the mutants (Fig. 2, E and F). In these mutant cell cultures, the individual cells appeared to be separated from each other. Even at high bacterial cell density, little or no star formation was apparent in the mutant cell populations.

Thus, it appears that all four saccharide-specific binding activities were concomitantly lost in the N4 and N6 mutants, lending support to the notion that these processes shared the same mechanism(s). In the accompanying manuscript (14), we document that the mutants N4 and N6 have also lost the BJ38 lectin activity, which was initially identified and purified from wild-type *B. japonicum*. All of these correlative data indicate that one likely candidate mediating these saccharide-specific binding activities is the BJ38 lectin.

Discussion

The data presented in this study document four saccharide-specific binding activities of intact *B. japonicum*: (a) binding

to polyacrylamide or Sepharose beads covalently derivatized with Lac; (b) homotypic star formation; (c) heterotypic binding to cultured SB-1 soybean cells; and (d) heterotypic adhesion to roots of soybean plants. All four binding activities shared the same carbohydrate specificity: Gal or Gal-containing glycoconjugates inhibited the interactions, but epimeric isomers failed to yield the same effect. Particularly striking was the exquisite specificity as seen in a comparison of the effects of Gal versus GalNAc. In all four assays, Gal showed inhibition, whereas its C-2 derivative did not.

Such a distinction between Gal and GalNAc calls attention to previous studies on the interaction of *B. japonicum* with root cells of leguminous plants, leading to the nitrogen-fixing symbiosis (1). Studies to account for the host specificity for compatible bacteria have been guided, for the most part, by the "lectin recognition" hypothesis, first proposed by Krüpe (15) and later revived by Hamblin and Kent (10) and by Bohlool and Schmidt (3). According to this hypothesis, legume lectins control host specificity by interacting with carbohydrate target molecules on the bacterial symbiont. In the soybean system, the primary candidate for the recognizer (lectin) on the host plant would be seed SBA (23) or the similar, if not identical, root lectin (6, 7, 31). The purified soybean plant lectin binds to both Gal and GalNAc. Although Stacey et al. (24) reported that both Gal and GalNAc inhibited *B. japonicum* binding to wild soybean (*Glycine soya*) roots, more recent studies by Vesper and Bauer (28) showed that inhibition was observed only with Gal, but not with GalNAc.

On the basis of direct observations of the initial binding as criteria, we have now confirmed that Gal, but not GalNAc, inhibits the adhesion of *B. japonicum* to soybean roots. The same specificity is apparent for the binding of the bacteria to cultured SB-1 cells. Again, while SB-1 lectin binds both Gal and GalNAc (19), the latter monosaccharide failed to inhibit *Rhizobium* binding (12, 13). This was the basis for our calling attention (12, 19) to the limitations of the lectin recognition hypothesis from the point of view that the plant cell accounted for the lectin, while the bacteria presented the carbohydrate.

An alternative view to the lectin recognition hypothesis, at least in the soybean-*Bradyrhizobium japonicum* interaction, is that the lectin resides on the bacteria while the plant cell presents the carbohydrate ligand. This view is supported by the identification, isolation, and characterization of a lectin from *B. japonicum*, which is reported in the accompanying paper (14). This lectin, designated as BJ38, exhibits the requisite specificity (i.e., it binds Gal with much higher affinity than GalNAc), consistent with the phenomenological observations documented in the present report.

Another line of evidence consistent with this notion comes from our analysis of two MNNG-derived mutants of *B. japonicum*, N4 and N6. These were selected on the basis of a decrease in one carbohydrate-specific binding property (adhesion to SB-1 cells), but were shown to be defective in all three of the other carbohydrate-specific binding activities as well. Although MNNG mutagenesis may cause multiple mutations, the concomitant loss of four binding properties through four independent mutation sites is rather unlikely. Moreover, biochemical isolation studies, detailed in the accompanying paper (14), failed to find the Gal-specific lectin activity corresponding to BJ38 of parental *B. japonicum* in the N4 and N6 mutants. Finally, preliminary evidence indi-

cates that the mutants N4 and N6 exhibit only 10–20% of the nodulation efficiency on soybean roots, as compared to wild type. These results strongly suggest that BJ38 may be a key component in mediating the common carbohydrate specificity of the various binding activities.

The localization of BJ38 on *B. japonicum* has not been determined. However, the facts that both homotypic star formation and heterotypic adhesion showed polar attachment of individual *Rhizobium* to other cells are suggestive that BJ38 may be a component of the fimbriae or pili, strandlike projections from the bacterial surface that have been the subject of many studies (27, 29, 30). In *R. lupini*, which forms star-shaped clusters of cells in autoagglutination, it was suggested that the pili may be responsible for cluster formation, as well as play a role in conjugation-mediated DNA transfer (11). Our present demonstration of saccharide-specific effects in autoagglutination of *B. japonicum* suggests that at least one component of the carbohydrate recognition system may be a substructure of the pili. In light of the polar orientation of bacterial attachment and the carbohydrate-specific binding activities of *B. japonicum* (adsorption to Lac-Sepharose and adhesion to soybean cells), it seems likely that the BJ38 molecule may be localized to the bacterial pili. This emerging view would be consistent with the work of Vesper et al. (29), who have purified pili from *B. japonicum*. Mutants of *B. japonicum* defective in attachment to soybean roots were shown to have a concomitant reduction in pilus formation (30). Thus, it was suggested that the pili of *B. japonicum* may be important mediators of bacterial attachment to soybean cells. Although no apparent sugar specificity has been demonstrated to be associated with the isolated pili, the identification of a lectin activity in the pili fraction would clearly be of interest.

Although we have emphasized the interaction of *B. japonicum* with soybean cells at the level of direct binding assays, our data should not be interpreted to preclude a role for the plant lectin in the complex pathway of nodulation and symbiosis. Stacey et al. have isolated *B. japonicum* mutants, after MNNG mutagenesis, that exhibit a delay in nodulation (8, 9, 25). These strains, SM1, SM2, and HS111, showed no defect in soybean root binding but were defective in the initiation of nodulation. Nodulation by HS111 can be facilitated by preincubation with soybean root exudates, SBA, or soybean root lectin. Thus, the plant lectin could facilitate infection by a mechanism other than soybean root binding. This notion is consistent with the observation that exogenous SBA, sufficient to saturate the SBA receptors on the bacterial cell surface, failed to inhibit *B. japonicum* binding to soybean roots (22). In addition, the transfer of the gene for pea lectin to clover plants allowed the successful infection of the normally noncompatible *R. leguminosarum*, leading to nitrogen-fixing nodules (5). Although the binding of *R. leguminosarum* to clover roots was not reported, these results nevertheless indicate that the plant lectins may play important roles during the infection process.

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