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Molecular Recognition Between *Bradyrhizobium japonicum* and Soybean Root*

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Abstract

Bradyrhizobium japonicum cells are capable of heterotypic binding to soybean roots and homotypic binding to other *B. japonicum* cells. Both of these binding phenomena showed the same saccharide specificity; lactose or galactose inhibited the binding, while N-acetyl-D-galactosamine did not. A carbohydrate-binding protein, BJ38, was isolated from *B. japonicum*. This protein was radioiodinated and examined for its saccharide-binding specificity to *B. japonicum* and to cultured soybean SB-1 cells. The saccharide specificity of BJ38 binding to SB-1 cells and to *B. japonicum* correlated with bacterial binding to soybean roots. Two binding-deficient mutants of *B. japonicum* were examined for the presence of BJ38 by immunoblot analysis. Both mutants were devoid of detectable BJ38 lectin activity. These results emphasized the significance of BJ38 in mediating the binding properties of *B. japonicum*. To evaluate the importance of the carbohydrate-binding properties of *B. japonicum* in the infection process, the ability of the mutants to nodulate soybean was compared to that of the wild-type bacteria in three ways: (1) dip inoculation; (2) direct inoculation; and (3) spot inoculation. In all three cases, the nodulation efficiency of the wild-type was much superior to that of the binding-deficient mutants. It is concluded that those bacteria possessing BJ38-mediated carbohydrate recognition may be more superior in binding to roots, resulting in better colonization and higher nodulation efficiency.

Keywords: *Rhizobium*, soybean, attachment, carbohydrate-binding protein, lectin

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1. Introduction

The binding of *Rhizobium* to legume roots has been studied extensively in symbiotic pairs, such as *Bradyrhizobium japonicum*-soybean, *Rhizobium leguminosarum*-pea and *R. trifolii*-white clover (for review, see Ho and Kijne, 1991; Ho, 1991; Halverson and Stacey, 1986; Dazzo and Truchet, 1983; Bauer, 1981; Dazzo et al., 1986). Several mechanisms have been proposed to be involved in the binding between the symbiotic bacteria and their hosts. The molecules thought to mediate this binding include plant lectins (Halverson and Stacey, 1986; Dazzo and Truchet, 1983), bacterial lectins (Ho et al., 1990a; Ho et al., 1990b), pili (or fimbriae (Vesper and Bauer, 1986; Vesper et al., 1987; Vesper and Bhuvaneswari, 1988)), rhicadhesins (Smit et al., 1989), and cellulose fibrils (Smit et al., 1986; Napoli et al., 1975). Rhicadhesins and cellulose fibrils have been demonstrated in several *Rhizobium* strains. The contribution of these molecules to bacterial attachment does not seem to be host specific.

B. japonicum binds to soybean roots in a carbohydrate-specific fashion. The plant lectin, soybean agglutinin (SBA), does not seem to play an important role in the binding (Ho and Kijne, 1991; Ho, 1992). This conclusion is derived from two key observations. First, a clear distinction in the saccharide specificity exists between SBA-ligand binding and *B. japonicum*-soybean interaction (Ho et al., 1988; Ho et al., 1990a; Vesper and Bauer, 1985). This bacterial strain is capable of attachment via a galactose (Gal)- or lactose (Lac)-specific recognition, such that Gal- or Lac-glycoconjugates can inhibit its attachment. However, N-acetyl-D-galactosamine, which is a potent hapten inhibitor for SBA, did not show any inhibition (Ho et al., 1988; Ho et al., 1990a; Vesper and Bauer, 1985). Second, *B. japonicum* can independently recognize glycoconjugates (Ho et al., 1990a). It can bind to beads derivatized with Lac and bind to other bacteria in a carbohydrate-specific manner (Ho et al., 1990a)

The carbohydrate-specific interaction between *B. japonicum* and soybean has been extensively reviewed (Ho and Kijne, 1991; Ho, 1992). This symbiotic interaction seems to be unique to the *B. japonicum*-soybean pair. No other *Rhizobium*-legume interaction, thus far, has been demonstrated to show the same carbohydrate recognition. The identification of the carbohydrate recognition of *B. japonicum* has extended our understanding of the molecular mechanism of how this microsymbiont interacts with its host, soybean (Ho et al., 1988; Ho et al., 1990a; Ho et al., 1990b) A major advance of this study was the purification of a carbohydrate-binding protein from *B. japonicum* cell extracts (Ho et al., 1990b). This protein was designated as BJ38, due to its molecular size of 38,000 and its origin from *B. japonicum*.

The present report is to demonstrate that purified BJ38 binds directly to

cultured soybean root cells of the line SB-1, and to other *B. japonicum* cells through a carbohydrate recognition similar to that of the *B. japonicum* binding to soybean roots. Thus, this protein may mediate the attachment of *B. japonicum* to soybean roots. The fact that two binding-deficient mutants of *B. japonicum* failed to express BJ38 binding activity further supports this notion. The importance of the binding property of *B. japonicum* to soybean roots in relationship to the infection process and nodulation is evaluated by comparing the nodulation efficiency of the binding-deficient mutants and the wild-type bacteria.

2. Materials and Methods

Bacteria and plant cell cultures

B. japonicum R110d and soybean SB-1 cells were cultured in YEMG and 1B5C media, respectively (Ho et al., 1988). The two binding-deficient mutants, N4 and N6, of *B. japonicum* were isolated after treatment of the wild type with N-methyl-N′-nitro-N-nitrosoguanidine (MNNG). They showed negligible binding to cultured soybean SB-1 cells, soybean roots, beads derived with Lac, and other *B. japonicum* cells (Ho et al., 1990a).

BJ38 isolation

The carbohydrate-binding protein BJ38 was isolated from 12 liters of *B. japonicum* cultures that had been grown to late exponential phase ($O.D._{620} = 1.7–2.0$). The cells were harvested, resuspended in 300 ml phosphate buffered saline (PBS, 10 mM sodium phosphate, 0.14 M NaCl, 4 mM KCl, pH 7.4), ruptured by a French press and fractionated by affinity chromatography on a column of Sepharose 4B derivatized with Lac (Ho et al., 1990b). BJ38 was eluted by 0.1 M Lac in PBS. The partially purified BJ38 was radioiodinated with $Na^{125}I$ by the chloramine-T method and was further purified by a second round of affinity chromatography. The Lac-eluted ^{125}I-BJ38 was dialyzed against 50 mM sodium phosphate buffer, pH 7.4, to remove the saccharide. The specific activity of the labeled BJ38 was about 65 μCi/μg protein. This ^{125}I-BJ38 sample was used for various binding studies.

Antibody directed against BJ38

Antibodies directed against BJ38 were generated by immunizing New Zealand white rabbits with purified BJ38. The immunogen was prepared by a two-step process. Partial purification of BJ38 was achieved by affinity chromatography on a Lac-Sepharose column. The BJ38 in the Lac-eluted fraction

was further purified by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). After the gel was stained with the silver reagent to reveal BJ38, the protein band corresponding to BJ38 was excised from the gel. The gel pieces (containing approximately 12 μg of BJ38) were homogenized with the adjuvant TiterMax (CytRx Corp., Norcross, Georgia, USA) and injected into a rabbit. Antiserum obtained 2 to 4 weeks after the immunization showed positive reaction with BJ38 as determined by immunoblot analysis.

Binding of ^{125}I-BJ38 to cultured SB-1 cells

Soybean SB-1 cells from a 2-day old culture were washed by centrifugation and suspension with MB5 medium (1B5C medium omitting 2,4-dichlorophenoxyacetic acid and casein hydrolysate). The cells were then resuspended in MB5 to a cell density of 10^6 cells/ml (1.0 ml of packed cells is approximately equal to 10^7 cells). Aliquots of 2.0 ml of cell suspension then were transferred to 12×75 mm culture tubes. After centrifugation (1 min at 1,500×g), the supernatant fraction was removed. Various saccharide solutions (0.1 ml) were added, followed by ^{125}I-BJ38 (1.2×10^4cpm in 0.1 ml). The samples were incubated with gentle shaking at room temperature for 4 hr. The samples were then washed three times with 2.0 ml MB5 by centrifugation (1 ml at 1,500×g) and resuspension. The radioactivity associated with the cell pellets was determined in a gamma counter (LKB, model 1271 Riagamma).

Binding of ^{125}I-BJ38 to B. japonicum *cells*

Cultures of *B. japonicum* at late exponential phase of growth were washed with YEMG medium once and resuspended in YEMG to a cell density of 10^8 cells/ml. Various saccharide solutions (0.1 ml) were added to 0.8 ml aliquots of bacterial suspension, followed by 0.1 ml of ^{125}I-BJ38 (1.2×10^4cpm). The samples were incubated at room temperature for 4 hr with gentle shaking. Individual samples were passed through membrane filters (0.22 μm), Millipore Corp., Bedford, MA (USA) that had been presoaked with 5% bovine serum albumin (BSA) in PBS to eliminate nonspecific binding. Bacteria retained on the membrane were washed three times with 2.0 ml of 0.1% BSA in PBS. The radioactivity bound to the bacteria was then determined.

Nodulation assay

1. *Dip inoculation.* Soybean seeds [*Glycine max* (L.) Merr. CV. Williams] were surface sterilized by soaking for 5 min in 1% sodium hypochlorite followed by 30 min in 3% hydrogen peroxide. Seeds were subsequently

given four 15 min rinses in sterile distilled water and then allowed to germinate for 4 days in the dark. The roots of the 4-day-old seedlings were dipped into a known concentration of *B. japonicum* for 5 hr and then washed three times by shaking in a beaker of sterile water. The seedlings then were planted in vermiculite in the greenhouse. The number of nodules developed was determined after 30 days.

2. *Direct inoculation.* Soybean seeds were surface sterilized and germinated in pots for 4 days. Bacterial suspension (50 ml, 10^7 cells/ml) was directly inoculated into the pot. The plants were grown for 30 days, and the number of nodules developed on each plant was determined.
3. *Spot inoculation.* Soybean seeds were surface sterilized, germinated, transferred to growth pouches in a growth chamber. Bacterial suspension (50 μl, 10^8 cells/ml) was sport inoculated directly onto the root system covering the area from the root hair region to the root cap as described by Bhuvaneswari et al. (1980). After inoculation, the plants were grown in the growth chamber for 14 days before the nodules were counted.

All nodulation experiments were performed at least three times with 30 to 40 plants for each bacterial inoculation.

3. Results and Discussion

B. japonicum cells were cultured to the late exponential phase of growth (absorbance at 620 nm = 1.7–2.0), at which time *B. japonicum* expressed maximum binding activity, as assayed on Lac-Sepharose beads. Cells were harvested and BJ38 was isolated by affinity chromatography on a Lac-Sepharose column. The initially isolated fraction, which was obtained after elution with 0.1 M Lac, contained multiple protein bands as indicated by SDS-PAGE and silver stain (Fig. 1, lane a). This fraction was radioiodinated by chloramine-T method, and repurified by a second affinity column. After these steps, ^{125}I-BJ38 was purified to homogeneity as determined by SDS-PAGE and autoradiography (Fig. 1, lane b). Silver stain of the sample after the second round of affinity chromatography yielded one single band corresponding to the band shown by autoradiography (Ho et al., 1990b).

If BJ38 mediated the bacterial autoagglutination and bacterial binding to soybean roots, the isolated polypeptide should be able to bind to soybean roots and to *B. japonicum.* This was tested by binding of ^{125}I-BJ38 to cultured soybean SB-1 cells and to *B. japonicum* (Table 1). ^{125}I-BJ38 bound to cultured soybean SB-1 cells and *B. japonicum* in a carbohydrate-specific manner, such

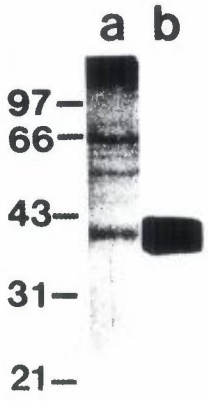

Figure 1. SDS-PAGE analysis of *B. japonicum* components after fractionation on a Lac-Sepharose affinity column. Lane (a), silver stain of a Lac-eluted fraction after initial isolation of BJ38; lane (b), autoradiography of the radioiodinated sample obtained from lane (a) and rechromatography on a second affinity column.

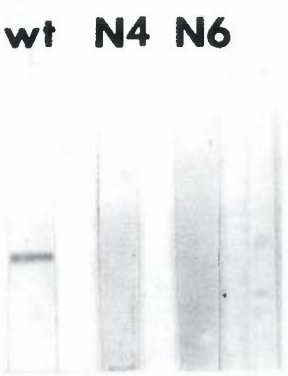

Figure 2. Immunoblot analysis of the Lac-eluted fractions isolated from affinity chromatography of *B. japonicum* extracts on a Lac-Sepharose column. Lanes: (wt) sample isolated from *B. japonicum* wild type; (N4) sample isolated from mutant N4; and (N6) sample isolated from mutant N6.

that Gal or Lac inhibited this binding. In particular, both N-acetyl-D-galactosamine (GalNAc) and galactosamine ($GalNH_2$), which are derivatives of Gal at the C-2 position, showed a drastic decrease in their inhibitory effect, relative to Gal. These results clearly demonstrated that ^{125}I-BJ38 was able to differentiate between Gal from other saccharides. More importantly, in both cases, ^{125}I-BJ38 bound to these cells with the same sugar specificity as those documented for bacterial autoagglutination and bacterial binding to soybean roots (Ho et al., 1988; Ho et al., 1990a). It is, therefore, suggested that BJ38 may mediate the carbohydrate-specific binding between *B. japonicum* and soybean roots.

Two binding-deficient mutants, N4 and N6, were isolated on the basis of their deficiency in binding to cultured soybean SB-1 cells (Ho et al., 1990a,b). These mutants showed a greatly diminished binding activity in all four binding assays: (a) soybean roots, (b) cultured soybean SB-1 cells, (c) Lac-Sepharose beads; and (d) other *B. japonicum* cells (Ho et al., 1990a). When these mutants were extracted for BJ38 isolation, after Lac-Sepharose affinity chromatography, no BJ38 could be detected from either mutant by SDS-PAGE and immunoblot

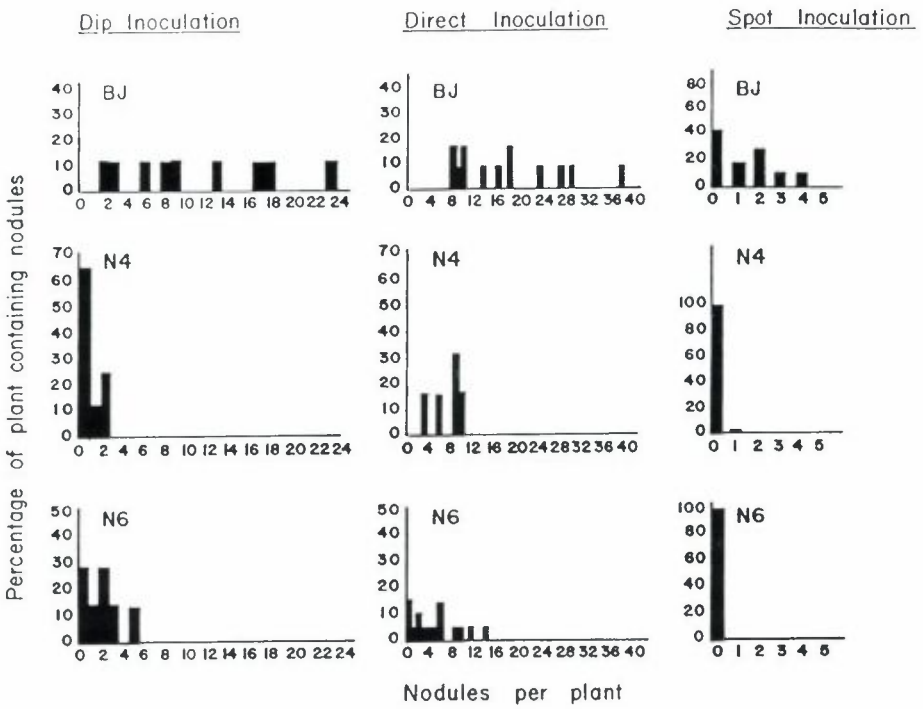

Figure 3. Comparison of nodulation activity of *B. japonicum* R110d and its mutants, N4 and N6. **Dip inoculation:** Roots of 4-day-old soybean seedlings were incubated with 10^8 bacteria/ml for 5 hr at room temperature and then washed three times with sterile water and planted on vermiculite. The number of nodules developed was determined after 30 days. **Direct inoculation:** Soybean seeds were germinated and grown in pots of vermiculite for 4 days. Bacterial suspension (50 ml, 10^7cells/ml) was directly inoculated into the pot. The plans were grown for 30 days and the number of nodules developed in each plant was determined. **Spot inoculation:** Soybean seedlings were grown in growth pouches in a growth chamber. Bacterial suspension (50 μl, 10^8 cells/ml) was spot inoculated directly onto the root system. After 14 days, the number of nodules in each plant was determined.

analysis with anti-BJ38 antibody (Fig. 2). These results indicate that mutants that were defective in expressing BJ38 binding activity were defective in all carbohydrate binding activities of *B. japonicum*. This conclusion indirectly supported the hypothesis that BJ38 is important in mediating all four binding properties of *B. japonicum*.

In order to evaluate the significance of carbohydrate-binding properties of *B. japonicum* in the infection process, the ability of the mutants to nodulate soybeans was compared to that of the wild-type in three ways: "dip" inoculation, "direct" inoculation and "spot" inoculation. The results of all three

Table 1. Effect of Saccharides on ^{125}I-BJ38 binding to cultured soybean SB-1 cells and *B. japonicum* cells*

Saccharide	SB-1 cells		*B. japonicum* cells	
	cpm bound	% binding	cpm bound	% binding
No hapten	3165±1025	100	11228±78	100
Clc	3150±509	99±16	10465±1449	93±12
Man	3290±127	103±4	11790±70	105±1
GalNAc	2895±558	91±17	11430±1173	101±10
$GalNH_2$	2790±70	88±2	10590±777	94±6
Mel	2215±601	69±18	8940±876	79±7
Gal	1300±212	41±6	5902±1014	52±9
Lac	1210±42	38±1	4300±113	38±1

* The binding assay was performed by incubating ^{125}I-BJ38 (1.2×10^4cpm) with SB-1 cells or *B. japonicum* cells for 4 hr at room temperature. The amount of ^{125}I-BJ38 associated with the cells was determined by radioactivity. The final concentration of saccharide used was 100 mM. The data represent the means of duplicates ± standard deviation.

assays are shown in Fig. 3. Irrespective of the assay, there is a clear distinction between the wild-type bacteria and the N4 and N6 mutants. In the spot inoculation assay, the low ability to induce nodules by wild-type bacteria may reflect the short period of 14 days used for the assay. For direct inoculation assay, some nodules were developed on the tap roots of soybean plants inoculated by the mutants. These mutants did not seem to be "delay" mutants, which acquire infectivity after host induction. These results can be simply interpreted by a decrease in nodulation efficiency of these binding-deficient mutants. Thus, these results confirm the notion, derived from our binding data (Ho et al., 1988; Ho et al., 1990a; Ho et al., 1990b), that carbohydrate recognition may be an important parameter in the infection and nodulation process. It can easily be rationalized that bacteria possessing BJ38-mediated carbohydrate recognition mechanism may have a selective advantage for binding to the roots more efficiently and, therefore, result in better colonization and higher nodulation efficiency.

The nodules induced by both types of mutants fixed nitrogen as determined by acetylene-reduction. Histochemical study of the nodules sections revealed normal nodule morphology. The mutant bacteria reisolated from the nodules displayed neither autoagglutination nor binding to SB-1 cells and soybean roots, suggesting that these bacteria were not revertants or contamination from the wild-type. The fact that these mutant strains still have the ability to carry on the rest of the normal infection pathway leading to the formation of nitrogen fixing nodules suggests that the chemical mutation may not affect

the components involved in the later development of nodulation. Therefore, it is likely that these mutant strains are mainly defective in the binding property, which is the basis for the phenotypic characteristic for selection of these mutants.

Vesper et al. had examined the mode of binding between *B. japonicum* and soybean (Vesper and Bauer, 1985, 1986; Vesper et al., 1987; Vesper and Bhuvaneswari, 1988). There are major differences between the results obtained from their work and ours. Vesper et al. proposed pili as the mediator for the attachment of *B. japonicum.* They isolated a pilus fraction containing two components with molecular weights of 18,000 and 21,000 (Vesper and Bauer, 1986). An antibody generated against this fraction labeled *B. japonicum* at one pole, blocked *B. japonicum* binding to soybean roots, and blocked the bacterial induced nodulation. The pili were also suggested to bind the bacteria to polystyrene plastic surfaces. No apparent specificity was assigned to these interactions. They also isolated two sets of *B. japonicum* variants, with pilus-negative phenotype; transposon inserted mutants (Vesper et al., 1987), and a field isolate (Vesper and Bhuvaneswari, 1988). These varians were shown to be decreased in polar attachment to soybean roots. However, there was no difference in their nodulation efficiency when compared with the wild-type *B. japonicum* 110 ARS.

The basis for the discrepancy between the present study and that of Vesper et al. is not apparent. However, there is a general consensus (Ho et al., 1988; Vesper and Bauer, 1985; Stacey et al., 1980) that *B. japonicum* binds to soybean roots through a carbohydrate-specific recognition. The present data clearly suggested that mutants without this carbohydrate recognition were inferior in inducing nodule formation. This indirectly emphasizes the importance of the BJ38-mediated carbohydrate binding in nodule initiation.

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