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CARBOHYDRATE BINDING ACTIVITES OF *BRADYRHIZOBIUM JAPONICUM:* LOCALIZATION AND EXPRESSION OF THE LECTIN BJ38

By

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ABSTRACT

CARBOHYDRATE BINDING ACTIVITIES OF *BRADYRHIZOBIUM JAPONICUM:* LOCALIZATION AND EXPRESSION OF THE LECTIN BJ38

By

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BJ38 is a lactose/galactose-specific binding protein that is proposed to mediate the attachment of *Bradyrhizobium japonicum* to soybean roots. Using a polyclonal antiserum generated against BJ38, the lectin has been unipolar localized on the bacterium cell surface. More importantly, BJ38 localization is coincident with the site of *B. japonicum* attachment to soybean cells. This result indicates that the distribution of BJ38 is consistent with its potential role in the polar adhesion of the bacterium to soybean roots.

The expression of BJ38, assayed at the polypeptide level, was found to be elevated when *B. japonicum* cells were cultured in the presence of saccharides. This induction effect was of the order lactose > galactose > mannose ~ no hapten, which is analogous to the relative affinities of these saccharides for BJ38. A similar effect was also observed at the mRNA level, using Northern blot analysis.

Treatment of *B. japonicum* cells with genistein also resulted in elevated levels of BJ38 at both the mRNA and polypeptide levels. In parallel, expression of $nodD_1$, the transcriptional regulator of the *nod* genes, was also found to be elevated by flavonoids in a fashion similar to that of BJ38 induction. These results raise the possibility that BJ38 may be a member of the *nod* gene family under the control of $nodD_1$.

Finally, the levels of $nodD_1$ transcription could also be elevated by saccharide treatment. This observation suggests that alternative inducers, besides flavoniods, may control the expression of the *nod* genes in *B. japonicum*.

To my parents

and

To Pearl

for their love and faith in me

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LIST OF ABBREVIATIONS

ATP	adenosine triphosphate
Api	apigenin
BJ38	carbohydrate binding protein 38 of *Bradyrhizobium japonicum*
Brj	*Bradyrhizobium japonicum*
BSA	bovine serum albumin
CBP	carbohydrate binding protein
cDNA	complementary deoxribonucleic acid
CPS	capsular polysaccharide
DOC	deoxycholate
DNA	deoxyribonucleic acid
FITC	fluorescein isothiocyanate
Gal	galactose
GalNAc	N-acetyl-D-galactosamine
Gen	genistein
Glc	glucose
kD	kilodaltons
Lac	lactose
LPS	lipopolysaccharide
Lut	luteolin
Man	mannose
mRNA	messenger RNA
Nar	naringenin
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffer saline
RNA	ribonucleic acid
SBA	soybean agglutinin
TBS	tris-buffer saline
TCA	trichloroacetic acid
YEG	yeast-extract-gluconate

CHAPTER I

LITERATURE REVIEW

INTRODUCTION

Rhizobia, belonging to the genera *Rhizobium, Bradyrhizobium* and *Azorhizobium,* are able to infect the roots of leguminous plants leading to the establishment of a root nodule symbiosis with the legume (1). Within the nodule, the bacteria differentiate into bacteriods that fix nitrogen which the plant uses as a nitrogen source. In return, the plant supplies the bacteria with carbon as energy sources. The infection process is characterized by a high degree of host-specificity (Table I). For example, *Rhizobium leguminosarum* bv. *viciae* (*R. leguminosarum*) nodulates only pea and vetch; *Rhizobium leguminosarum* bv. *trifolii* (*R. trifolii*) nodulates clover; and *Bradyrhizobium japonicum* (*B. japonicum*) nodulates soybean. This host specificity is observed throughout the nodulation process, including stages prior to the initiation of infection. Several steps or levels of recognition appear to be involved in this host-specific nodulation process, and these include the interaction between components derived from both the bacteria and the plant symbiont (2-4).

At the first level, there are diffusable signals secreted from the plant to the bacteria. These signals include phenolics and flavonoid compounds, the latter of which are products of the phenylpropanoid pathway and almost ubiquitous in the plant kingdom (5). Rhizobia are chemotactic toward these flavonoid compounds (6-9).

Table I. Classification of Rhizobia and Their Symbiotic Hosts

Genus	Members	Common Hosts
Rhizobium	*R. fredii*	Wild soybean
	R. leguminosarum	
	biovar *phaseoli*	Bean (Phaseolus)
	biovar *trifolii*	Clover (Trifolium)
	biovar *viciae*	Pea (Pisum); Vetch (Vicia)
	R. loti	Trefoil (Lotus)
	R. meliloti	Alfalfa (Medicago)
Bradyrhizobium	*B. japonicum*	Soybean (Glycine)
	B. lupini	Lupine (Lupinus)
Azorhizobium	*A. caulinodans*	Sesbania (stem-nodulating)

The secreted flavonoids are plant-specific (10-12) and are able to stimulate the coordinate transcription of an important set of bacterial genes (*nod* genes) that are necessary for nodulation. This process of induction by flavonoids is mediated by NodD, a transcriptional activator protein encoded for by the *nodD* gene (13-15). The expression of *nodD* varies among rhizobial species; it is induced by flavonoids in *B. japonicum*, autoregulated in *R. leguminosarum*, and constitutively expressed in *Rhizobium meliloti (R. meliloti)* (13-14, 16). In the presence of flavonoids, the NodD protein binds to the conserved *nod* box DNA sequence preceeding the *nod* genes and activates the transcription of these genes (17-20). This interaction of the NodD protein with flavonoids is specific in that the NodD proteins from different species of bacteria are able to recognize only specific flavonoids (11, 21). Thus, genistein, a specific inducer of *nod* genes in *B. japonicum* only interacts with the NodD protein of *B. japonicum* (12, 16) while luteolin, the equivalent inducer in *R. meliloti,* only activates the NodD protein in that species (10, 13). In this connection, it has also been demonstrated that the transfer of a *nodD* gene from *Rhizobium sp.* NGR234 into other *Rhizobium* strains leads to extended host range for the *nodD* gene recipient (22).

Flavonoids can also serve as repressors of *nod* gene expression in non-symbiotic strains. For example, diadzein and genistein, which are strong inducers in *B. japonicum*, are potent inhibitors of gene expression in *R. leguminosarum* and *R. meliloti* (23, 24). The molecular recognition process involving flavonoids, therefore, constitutes an important first level determinant of host-*Rhizobium* specificity (2-4).

The second level of recognition involves diffusible signals from the bacterium to the plant that are termed Nod factors. The Nod factors are able to initiate many of the early nodulation responses in the plant symbiont. For example, alfalfa roots exposed to the Nod factors from *R. meliloti* demonstrate membrane depolarization (25) and morphogenic changes associated with the infection process; these changes include root-hair deformation, curling and the formation of pseudo-nodules (26, 27). The Nod factors are synthesized by proteins that are the product of the *nod* genes. These *nod* genes fall into two general groups; the common *nod* genes (*nodABC*) that are essential for the nodulation of any host, and the host specific *nod* genes which are rhizobium-strain specific (2, 4, 28). This latter group of *nod* genes determine host specificity by species-specific chemical modification of the β-1,4 linked N-acetyl-D-glucosamine sugar backbone of the Nod factor. Thus the addition of a sulfate group to the sugar backbone by the *nodHPQ* gene products is an important determinant of host specificity of the *R. meliloti* factor (27-29), while the O-acetyl group and nature of the fatty acyl substituent affect the biological activity of the *R. leguminosarum* factor (30). Mutations to these host-specific *nod* genes can lead to structural changes of these signals resulting in a change in the host range of the bacterium. For instance, a *nodH* mutant in *R. meliloti* produces a non-sulfated Nod factor and is unable to nodulate alfalfa; instead, it has gained the ability to nodulate vetch, a *R. leguminosarum* symbiont (31, 32).

The third level of recognition occurs at the attachment of rhizobia to the root surface. Rhizobia are found to bind predominantly to the root hairs and it is in this

region that the infection process begins (33). Inoculation of rhizobia with seedling roots leads to an initial clumping and binding of bacterial cells at the tips of the roots. Rhicadhesin (M_r 14,000), a Ca^{2+} binding protein is proposed to mediate this initital attachment (34). Rhicadhesin is a *nod* gene independent bacterial surface protein found in most rhizobial strains (35). A second binding phase follows in which the bacteria begin to attach to the root tips in an end-to-end (polar) fashion. This second phase results in firm attachment of rhizobia to the legume roots (36) and is proposed to control the host-specific interaction of rhizobia with its symbiont (37,38). Much of our understanding of how this host-specific attachment occurs has been guided by the lectin-recognition hypothesis proposed by Krüpe (39). In this hypothesis, legume lectins are proposed to control the specificity of the rhizobia-legume attachment by specifically interacting with saccharide components on the bacterial cell surface. This hypothesis is discussed in the context of the following rhizobial-legume systems.

(i) ***R. leguminosarum* bv. *trifolii*-clover system**

The clover-*R. trifolli* symbiosis provides the most convincing evidence of the role of the plant lectin in controlling the host specific attachment of rhizobia to the legume root. Trifoliin A, a clover lectin (M_r 50,000) that binds to 2-deoxyglucose has been identified and proposed to function in this role (40). Trifoliin A is multivalent in binding to and can agglutinate *R. trifolii*. Infective strains of *R. trifolii* contain polysaccharides that are antigenically cross-reactive with the clover root hair cell surface (41). This cross-reactive antigen (CRA) binds to trifoliin A as well as to root cells in a fashion matching the distribution of the lectin. The clover root lectin

thus functions as a cross-bridge between *R. trifolii* and the root hair. Consistent with this idea, the sugar 2-deoxyglucose can specifically inhibit binding of *R. trifolii* or its capsular polysaccharide to clover root hairs (42). A strong correlation also exists between lectin binding and infectivity of *R. trifolii* (40,43). When the expression of lectin receptors on the bacterial cell surface as a function of culture age were examined, the number of lectin receptors on *R. trifolii* directly correlated with the number of cells bound to the clover root hairs (44). Hence, for the *R. trifolii*-clover symbiosis, all the presently available evidence lend support to the lectin-recognition hypothesis.

(ii) ***R. leguminosarum* bv. *viciae*-pea system**

The attachment of *R. leguminosarum* to pea roots appears to be mediated by rhicadhesin, as evidenced by the ability of purified rhicadhesin to inhibit rhizobial attachment to pea roots (35). A pea lectin is also present in the roots of the pea legume. However, unlike the *R. trifolii*-clover symbiosis, the plant lectin does not appear to play a direct role in the attachment process. This belief arises from the observation that rhizobial attachment cannot be inhibited by saccharides, including affinity haptens (mannose (Man) and glucose (Glc)) of the pea lectin. Moreover, on surveying the distribution of the pea lectin, the lectin was found on the external surface of the plasma membrane rather than the outer surface of the plant cell wall where it could mediate attachment (45).

The pea lectin, though not directly involved in the attachment process, nevertheless can enhance nodulation as the secreted lectin can contribute to the

accumulation of rhizobia at the root hair tips (46). The most striking and persuasive evidence for a role of the pea lectin in root infection is the finding that transfer of the *psl* gene coding for the pea lectin to white clover roots resulted in nodulation of the transgenic roots by *R. leguminosarum* (47). Normally, white clover roots release appropriate flavonoids for the induction of *R. leguminosarum nod* genes and signal molecules produced by the bacteria are recognized by the clover root hairs (21, 48). However, root hair curling is usually abnormal and infection threads are not found. Transformation of white clover roots with the pea lectin gene, however, conferred upon these roots the ability to be nodulated by *R. leguminosarum.* Thus it appears that the pea lectin might indeed play a key role in the normal pea-*R. leguminosarum* symbiosis.

(iii) *B. japonicum*-soybean system

A plant lectin has also been demonstrated to be present in soybean roots. This lectin, isolated on the basis of its carbohydrate-binding activity, exhibits molecular and saccharide binding properties similar to seed soybean agglutinin (SBA) (49-51). All available evidence, however, fails to demonstrate that the plant lectin actually plays an indispensable role in the recognition process. This viewpoint stems from the following observations (52). First, the binding of SBA to soybean nodulating bacteria is not selective in that SBA does bind to certain rhizobial strains that do not infect soybean roots. Second, some *Rhizobium* strains that nodulate soybean roots do not bind detectable amounts of SBA. Third, soybean roots that lack SBA are still able to

form root nodules. Fourth, exogenously added SBA, at a concentration sufficient to saturate the *Rhizobium* cell surface, resulted in only 35% inhibition of binding to soybean roots (53). Fifth, there is a glaring discrepancy in the saccharide specificity of SBA, and the specificity of saccharide inhibition of *B. japonicum* binding to soybean roots. If indeed SBA mediates attachment of *B. japonicum* attachment to soybean roots, then the addition of SBA haptens galactose (Gal) and N-acetyl-D-galactosamine (GalNAc) would inhibit bacterial attachment. However, studies by Vesper et al. (54) and Ho et al. (55) have shown that bacterial binding was only inhibited by Gal but not GalNAc. The specificity of saccharide inhibition of *B. japonicum* attachment is not consistent with SBA's proposed role in mediating the bacterial attachment to its symbiont. This saccharide specificity in itself may reflect recognition components, other than the plant lectin, which exhibit Gal-specific characteristics.

Finally, the distribution of SBA binding polysaccharides is incompatible with its role in mediating *B. japonicum* attachment. Studies by Tsien et al. (56) have revealed two distinct poles in *B. japonicum:* the nucleoid portion containing the cytoplasm and the bacterial chromosome, and the reserved polymer end which contains hydroxybutyrate and glycogen granules. The SBA-binding polysaccharides are localized in the nucleoid portion (57). As the nucleoid end of the bacterium is the portion of the bacterium away from the point of attachment, it is highly unlikely that the plant lectin can play a direct role in bacterial attachment.

Similar to the pea lectin of *R. leguminosarum*-pea interaction, SBA may still

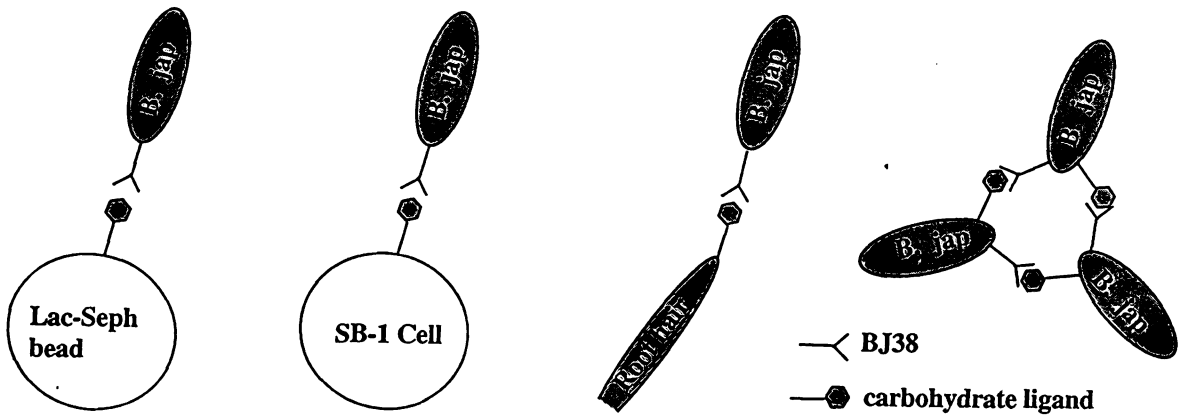
have a role to play in the nodulation process. SBA can interact with the bacterial surface and initiate metabolic responses (58-60). A mutant of *B. japonicum* that exhibits a delay in nodulation can be reversed by pretreating these cells with SBA or soybean root exudate (60). This effect is specific in that it could not be repeated using root exudates or lectins from other legume species. In addition, SBA could also enhance the nodulation efficiency in wild-type bacteria, facilitating nodule initiation at a low inoculum of bacterium (60). These observations clearly demonstrate that though the plant lectin performs no direct role in the attachment of bacterium to the soybean root, it does participate in facilitating the nodulation process.

Saccharide-specific binding activities of *B. japonicum*

In the course of studies to characterize the rhizobium-soybean interaction, it was discovered that *B. japonicum* exhibited four carbohydrate-binding properties: (i) heterotypic binding to soybean root; (b) heterotypic adhesion to cultured soybean SB-1 cells, originally derived from soybean roots; (c) homotypic autoagglutination; and (d) adsorption to sepharose beads derivatized with lactose (Lac-Seph) (55). All of these binding properties are inhibited by Gal, but not by GalNAc. In light of the similar saccharide specificities of carbohydrate inhibition, it was proposed that these four assays may be mediated by the same mechanism or components. This hypothesis is diagrammed schematically in Fig. 1. The key feature of these models is that in each of the cases of heterotypic binding, the lectin resides on the bacterium, while the

Figure 1. Models illustrating the role of BJ38 in the binding of *B. japonicum* to: (a) Lac-Sepharose beads; (b) SB-1 cultured soybean cells; (c) soybean roots; and (d) other *B. japonicum* cells, leading to autoagglutination.

(a)


B. jap
Lac-Seph bead
B. jap
SB-1 Cell
B. jap
Root hair
B. jap
B. jap
B. jap
BJ38
carbohydrate ligand

carbohydrate ligand is presented by the other partner. In the case of homotypic agglutination, both the lectin and carbohydrate ligand are found on the bacterial surface. Consistent with this notion, a carbohydrate-binding protein, BJ38 (M_r 38,000) was purified from *B. japonicum* cells. BJ38 bound specifically to Gal and Gal-containing sugars, but not to the epimeric sugars mannose (Man) or glucose (Glc) (61). In addition, a modification of Gal at the C-2 position resulted in a drastic lowering of affinity as demonstrated by an 18-fold reduction of binding by GalNAc. The carbohydrate-binding specificity of BJ38 was, therefore, in good agreement with the specificity of saccharide inhibition of *B. japonicum* binding in the four saccharide-specific assays. BJ38 is thus a likely candidate responsible for mediating the saccharide-specific binding of *B. japonicum*.

This hypothesis was further tested with mutants that were defective in attachment. If indeed BJ38 was responsible for mediating the carbohydrate-specific binding in all four assays, two observations would follow. First, mutants isolated on the basis of an impairment in one of the binding activities should show a concomitant loss of the other binding activities. Second, the mutants would produce little or defective BJ38 compared to the parental strain. Chemical mutagenesis of wild-type bacteria with N-methyl-N'-nitro-N-nitrosoguanidine, followed by selection based on reduced binding to soybean SB-1 cells, resulted in the isolation of two mutants, N4 and N6 (55). Compared to the wild-type, these mutants showed no difference in morphology, growth patterns and staining with both SBA, and antibodies generated against the lipopolysaccharide and capsular polysaccharide fractions of *B. japonicum*

(anti-Brj). However, when the mutants were analyzed for the ability to participate in all four assays, absent or reduced binding was observed. When extracts from the N4 and N6 mutants were fractionated over a Lac-Seph column, no BJ38 could be identified. These results further support the hypothesis that the saccharide binding properties are mediated by a common mechanism/components and that BJ38 may be directly involved in the attachment process.

The importance of BJ38 and bacterial binding in the infection process was investigated by comparing the nodulation efficiency of the mutants with the wild-type bacteria. When soybean seedlings were inoculated with three doses each of wild-type, N4 and N6 bacteria, the plants treated with the mutant cells showed a reduction in nodule formation, relative to the parental strain (62). This result suggests that the carbohydrate-specific binding process, and possibly BJ38, may be an important component in the symbiosis between soybean plants and *B. japonicum.* Consistent with this, purified BJ38 was found to bind predominantly to the young emergent root hairs of soybean roots and to a much lesser extent, to the root cap, mature root hairs, epicotyl or hypocotyl region. This positional specificity was identical to that observed in *B. japonicum* cell binding to the soybean root. In previous studies, it has been documented and postulated that rhizobia tend to colonize at the root hair zone as the rhizosphere surrounding it were rich in nutrients suitable for bacterial viability and growth. Nonetheless, this reasoning could not account for the preferential binding of BJ38 to these regions, as the lectin's binding is not dependent on nutrients and factors of the environment. Thus, it seems more likely that both *B. japonicum* and BJ38 bind

bind selectively to the emergent root hairs as these regions express the carbohydrate structures recognized by BJ38. In studies by Bauer and coworkers (63, 64), it was also demonstrated that the emergent root hair zones were regions of the soybean root that were most susceptible to root-hair deformation, a key step in the infection process leading to the formation of nitrogen fixing nodules. Thus three key correlations are observed which support the hypothesis that BJ38 mediates *B. japonicum* binding to soybean roots and that the attachment is a significant step leading to successful nodulation. First, *B. japonicum* cells preferentially bind to the young, emergent soybean root hairs; second, BJ38 binds to sites that are coincident with bacterial binding; third, these sites have been shown to be most susceptible to infection and nodule initiation.

In summary, though the lectin-recognition hypothesis appears to be upheld in the *R. trifolii*-clover system, this hypothesis has fared less well in the pea and soybean systems. The documentation of a carbohydrate specific lectin isolated from *B. japonicum* presents a new and alternative view to the lectin-recognition hypothesis; in that the bacteria provides the protein and the plant the carbohydrate.

In this dissertation, I describe the characterization of BJ38 in terms of its distribution in *B. japonicum*. This determination is important as the *B. japonicum* cells bind to soybean roots in a polar fashion. Thus any hypothesis implicating a role for BJ38 in bacterial binding will require the lectin to be exposed at the cell surface where the attachment occurs. In addition, I also describe the characterization of BJ38 in terms of factors that regulate its expression. The infection process occurs via a

series of steps that involve the activation of *nod* genes. As the attachment process, possibly involving BJ38, is a requisite step leading to the formation of nitrogen fixing nodules, the possibility exists that BJ38 may be a member of the *nod* gene family. This possibility is studied in terms of the ability of specific inducers of the *nod* genes in *B. japonicum* to similarly induce BJ38.

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CHAPTER II

CARBOHYDRATE BINDING ACTIVITIES OF *BRADYRHIZOBIUM JAPONICUM*

Unipolar Localization of the Lectin BJ38 on the Surface of *B. japonicum*

FOOTNOTES

[1]This work was previously published in the Proceedings of National Academy of Science (1993) 90:3033-3037 and is reproduced with permission from the publisher.

[2]The electron microscopy work was performed by Dr. Siu-Cheong Ho.

ABSTRACT

A polyclonal antiserum generated against the *Bradyrhizobium japonicum* lectin BJ38 was characterized to be specifically directed against the protein. Treatment of *B. japonicum* cells with this antiserum and subsequent visualization with transmission electron and fluorescence microscopy revealed BJ38 at only one pole of the bacterium. BJ38 appeared to be organized in a tuft-like mass, separated from the bacterial outer membrane. BJ38 localization was coincident with the attachment site for: a) homotypic agglutination to other *B. japonicum* cells; b) adhesion to the cultured soybean cell line, SB-1, and c) adsorption to Sepharose beads covalently derivatized with lactose. In contrast, the plant lectin soybean agglutinin labeled the bacteria at the pole distant from the bacterial attachment site. The results documented indicate that the topological distribution of BJ38 is consistent with a potential role for this bacterial lectin in the polar binding of *B. japonicum* to other cells and surfaces.

INTRODUCTION

Many eukaryotic and microbial cells can recognize and interact specifically with other cells, either cells of the same type (homotypic) or cells of a different type (heterotypic). One example is the binding of the bacterium *Rhizobium* to the root cells of leguminous plants, leading to the formation of the symbiotic association and nitrogen fixing nodules (1,2). Several lines of evidence indicate that one key event in the establishment of such a symbiosis is the initial binding between the bacterium and the host cell (3).

In previous studies (4), we have documented that *Bradyrhizobium japonicum*, which normally infects the roots of soybean plants, exhibits four saccharide-specific binding activities: (a) adsorption to Sepharose beads derivatized with lactose (Lac-Sepharose); (b) homotypic autoagglutination; (c) heterotypic binding to cultured soybean (SB-1) cells; and (d) heterotypic adhesion to soybean roots. In all four of these assays, galactose inhibited the binding but a C-2 derivative of the monosaccharide, N-acetyl-D-galactosamine, failed to yield the same effect. When mutants of *B. japonicum*, designated as N4 and N6, were isolated on the basis of a defect in one binding activity (SB-1 cell binding), it was found that they showed a concomitant loss in the other three binding capacities (4). These observations suggested that all four of the carbohydrate-specific binding processes may be mediated by the same component(s) and mechanism(s). Consistent with this notion, we have purified a carbohydrate-binding protein, designated BJ38 (M_r ~38,000), which has an affinity for

galactose and Lac about 13- and 240-fold higher, respectively, than that for N-acetyl-D-galactosamine (5). This carbohydrate binding specificity correlates well with that of the *B. japonicum* binding activities. It was proposed, therefore, that BJ38 may mediate the carbohydrate binding activities of *B. japonicum*.

In all the four carbohydrate-specific activities tested, *B. japonicum* bound in a polar fashion (4). Thus, any hypothesis implicating a role of BJ38 in these binding assays would require that the lectin must not only be anisotropically localized at the pole of the bacterium, but also must be exposed at the surface of the bacterium where attachment occurs. In the present communication, we report on the characterization of a specific anti-BJ38 antibody that has allowed us to identify the topological distribution of BJ38 on the bacterial cell surface.

MATERIALS AND METHODS

Cell Cultures

B. japonicum (R110d) was originally obtained from Dr. Barry Chelm of Michigan State University. This bacterial strain was maintained on agar plates containing yeast extract-sodium gluconate medium (YEG) and 50 mM lactose. YEG contained (per liter): 1.28 g K_2PO_4, 0.2 g $Mg_2SO_4.7H_2O$, 7.35 mg $CaCl_2.2\ H_2O$, 28 mg sequestrene, 5.0 g gluconic acid, and 1.0 g yeast extract, and was supplemented with trace elements: 2.5 mg Na_2EDTA, 4.39 mg $ZnSO_4.7\ H_2O$, 0.77 mg $MnSO_4.H_2O$, 0.15 mg $CuSO_4.5\ H_2O$, 2.49 mg $Na_2MoO_4.2\ H_2O$, 0.23 mg $CoCl_2.6\ H_2O$, 0.46 mg $Na_2B_4O_7.10\ H_2O$, 0.38 mg Na_3VO_4, and 0.1 mg $NaSeO_3$, pH 6.0. The bacteria (3-day-old) were transferred from the agar plate to 50 ml YEG in a 125 ml Erlenmeyer flask and cultured for one day. The bacterial suspension was then inoculated into 2 liters of YEG and cultured for 2 days on a gyratory shaker (120 rpm) at 30°C. Aliquots of this culture (300 ml) were then inoculated into six Fernbach flasks, each containing 1.5 liters of YEG. The bacteria were further cultured for about 30 h until a value of 1.7-2.0 was obtained for the absorbance at 620 nm (A_{620}). The bacterial cells were then used for the isolation of BJ38, capsular polysaccharide (CPS), lipopolysaccharide (LPS), and for immunolocalization studies.

The SB-1 cell line, derived from soybean roots (*Glycine max* (L) Merr. cv. Mandarin) was kindly provided by Dr. G. Lark (Department of Biology, University of Utah, Salt Lake City). Cultures were grown in 50 ml of 1B5C medium at 27°C in

a gyratory shaker in the dark as described before (6). Continuous cultures were maintained by subdividing cells every 4 days, transferring 15 ml of culture to 50 ml of fresh 1B5C.

Isolation of BJ38

The bacterial culture (A_{620} = 1.7) was centrifuged at 8,000 rpm for 15 min in a Sorvall GS-3 rotor. The pelleted bacteria were frozen at -20° C overnight, thawed and then subjected to a passage through the French press at 1,600 psi. The bacterial cell extract was then centrifuged at 15,000 rpm in a Sorvall SS-34 rotor for 30 min and the supernatant was subjected to affinity chromatography on Sepharose covalently derivatized with lactose (Lac-Sepharose) at 4° C. The column (1.5 x 7 cm) was prepared by coupling lactose to activated Sepharose 4B beads using the procedure of Matsumoto *et al.* (7) and equilibrating with PBS (10 mM sodium phosphate, 0.14 M NaCl, 4 mM KCl, pH 7.4). After loading the extract, the column was washed with 300 ml of PBS and material bound to the column was then eluted with 50 ml of 0.1 M Lac in PBS. Fractions containing the lectin were identified by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using 10% acrylamide gels (8). Polypeptides were revealed by silver staining (9) or by immunoblotting.

For immunoblotting, proteins were transferred to the Immobilon-p (Millipore, Naperville, IL) membrane by electrophoresis (400 mA, 2 h, 25° C). The membrane was blocked overnight in TBS (20 mM Tris, 500 mM NaCl, pH 7.5) containing 5% (wt/vol) bovine serum albumin (BSA). Membranes were then incubated with 1:20

dilutions of the primary antiserum for 8 h and then washed three times with 10 min incubations in TTBS (TBS containing 0.05% Tween 20). The membranes were incubated with a 1:1000 dilution of goat anti-rabbit IgG coupled to alkaline phosphatase in 5% BSA/PBS for 1 h at 25° C, followed by washing in TTBS (3 times, 10 min each). The immunoreactive material was then developed with nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate as substrates.

Antibody Reagents

Fractions containing BJ38 lectin isolated from 100 liters of bacterial culture were concentrated by acetone precipitation (10). The BJ38 fractions were mixed with ice-cold acetone at a ratio of 4:1 (v/v) and incubated in a dry-ice/methanol bath for 30 min before centrifugation at 10,000 rpm using a SS-34 rotor. The pellet was resuspended in SDS sample buffer, and analyzed by silver staining after SDS-PAGE (10% acrylamide). Gel slices containing BJ38 lectin were excised. This sample, containing about 10 μg of BJ38, was homogenized with Titer-Max adjuvant (CytRx Corporation, Norcross, GA) and injected into a female New Zealand White rabbit. Booster injections of 6 μg of BJ38 in Titer-Max adjuvant were administered every three weeks. Blood from the rabbit was collected one week after each boost. The antiserum was collected and stored at -20° C in 1 ml aliquots.

To obtain monospecific antibodies directed against BJ38 from the antiserum, BJ38 fractions eluted from the Lac-Sepharose column were subjected to SDS-PAGE and immunoblotted with anti-BJ38 antiserum. A strip of the blot was cut out and the

position of migration of BJ38 was determined by developing the immunoblot. Strips corresponding to BJ38 were then cut out from the remainder of the blot and antibody specific to BJ38 was eluted by 0.1 M glycine, pH 2.3 for 30 sec with vortexing, according to the method of Smith and Fisher (11). The eluant was neutralized with 2 M Tris, pH 7.5. This treatment was repeated 5 times, and the eluant was pooled together and dialyzed against PBS overnight. The dialysate was lyophilized and the monospecific, affinity purified anti-BJ38 antibody was resuspended in water.

Purification of CPS and LPS

The CPS fraction of *B. japonicum* was isolated by the method described by Mort *et al.* (12). The bacterial culture (A_{620} = 1.7) was centrifuged at 8,000 rpm for 15 min in a Sorvall GS-3 rotor. CPS was then removed from *B. japonicum* by a 2 min treatment in a Waring blender. These cells were centrifuged at 15,000 rpm for 30 min in a Sorvall SS-34 rotor. The supernatant was dialyzed 24 h against water and the dialysate lyophilized.

The LPS fraction was purified from *B. japonicum* by phenol-water extraction (13). The pelleted bacteria were first washed with 0.5 M NaCl three times to remove exopolysaccharide. Equal volumes of water and phenol was added to the cells and the mixture incubated at 65°C for 15 min. The aqueous layer was collected and dialyzed 24 h against distilled water. The dialysate was passed over a Dowex AG 1X8 (acetate form) (Sigma, St. Louis, MO) column. The flow-through fraction containing LPS was lyophilized.

Fluorescence Studies

a) *B. japonicum* cells in suspension culture: *B. japonicum* cells (A_{620} = 1.7) were fixed onto coverslips according to the method of Aplin and Hughes (14). The cells were blocked for 2 h in 5% (wt/vol) BSA in YEG. One set of the sample was incubated with fluorescein (FITC) labeled soybean agglutinin (SBA; Sigma) for 30 min, followed by washing (2 times, 10 min each) in PBS. The remainder of the sample was incubated for 4 h with 1:20 dilutions (in 5% BSA/YEG) of preimmune serum and anti-BJ38 of this study and anti-Brj raised against heat-killed *B. japonicum* cells (6). After washing in YEG (3 times, 10 min each), FITC-conjugated goat-anti rabbit antibody (1:50 dilution, Boehringer Mannheim Biochemicals, Indianapolis, IN) in 5% BSA/YEG was added and incubated for 1 h. The samples were washed with PBS to remove the unbound antibody. Stained cells were then visualized with a fluorescence microscope (Carl Zeiss D-7082 Oberkochen; excitation filter: 546 nm; chromatic beam splitter: 580 nm; barrier filter: 590 nm).

b) Autoagglutination of *B. japonicum* that exhibited star formation: *B. japonicum* cells were grown on a YEG agar plate for 5 days. These cells were then fixed onto coverslips according to the method of Aplin and Hughes (14). The cells on the coverslips were blocked in 5% BSA/YEG for 2 h. The antibody or SBA labeling procedure was performed as in (a).

c) *B. japonicum* binding to SB-1 cells or Lac-Sepharose beads: *B. japonicum* cells (A_{620} = 1.7) were incubated with either SB-1 cells (2-day-old) or Lac-Sepharose beads in a petri dish for 3 h. The mixture was then transferred to 5 ml culture tubes

to allow the bound fraction to settle to the bottom of the tube. Unbound *B. japonicum* cells were removed and the bound cells were fixed in 2% paraformaldehyde in YEG for 30 min. The cell cultures were subjected to three washes in YEG (10 min each) and blocked in YEG containing 5% (wt/vol) BSA for 2 h. The *B. japonicum* cells that were bound to SB-1 cells were treated with either FITC-SBA or anti-BJ38 antiserum, while the *B. japonicum* cells bound to Lac-Sepharose beads were treated with only anti-BJ38 antiserum. The SBA and antibody labeling procedures were then performed as above in (a).

Electron Microscopy

Both *B. japonicum* cells from suspension culture (similar to the immunofluorescence studies) and actively growing cells in solid agar YEG medium (a 3-day-old culture in which a majority of the cells participated in star formation) were used. For *B. japonicum* grown on agar plates, the cells were first suspended in 2 ml of YEG medium. Cell clumps were dispersed by resuspension with a Pasteur pipette. The star-forming clusters or single cells from suspension culture were fixed for 15 min on ice in 0.25 % glutaraldehyde (EM Grade, Electron Microscopy Sciences, Fort Washington, PA) in YEG. The cells were then washed with PBS by centrifugation (3,000 rpm, HL-4 rotor, 15 min) and resuspension. The cell pellets were suspended in BSA-PBS to inactivate the remaining aldehyde groups of the fixative. After 1 h on ice, the samples were centrifuged (3,000 rpm, HL-4 rotor, 15 min) and the pelleted cells were suspended in anti-BJ38, anti-Brj or normal rabbit sera (1:20 dilution in

BSA/PBS, 4 h on ice). The samples were then washed three times with 4 ml PBS and labeled with goat-anti-rabbit IgG coupled with colloidal gold (1:5 dilution, particle size of 20 nm, Polysciences, Warrington, PA). After incubation for 1 h at room temperature, the cells were washed three times and resuspended in 4 ml PBS, and stored at 4° C overnight. The bacteria were then centrifuged at 3,000 rpm for 15 min in a HL-4 rotor and the pelleted cells were resuspended in 0.2 ml of PBS before being applied to the grids.

The formvar coated grids were first treated with 1 N HCl (5 min) to provide a negatively charged surface. After washing two times with water, the grids were then coated with 1 % polylysine (M_r 100,000, Sigma) in water for 5 min. Excess amounts of polylysine were removed by rinsing in water three times. A drop of the bacterial sample was then applied on each grid and the cells were allowed to settle on the grid for 15 min. Excess sample was blotted dry. The grids were washed three times with water to remove residual salt. The samples were then either directly observed under an electron microscope or stained with 1 % phosphotungstic acid, pH 7.2 before observation.

RESULTS

Antibodies Directed Against BJ38

BJ38 was purified by affinity chromatography on Lac-Sepharose and subjected to SDS-PAGE. The gel slices corresponding to the M_r 38,000 region were used as the immunogen for generating a rabbit antiserum against the lectin. The resulting antiserum, designated rabbit anti-BJ38, yielded a single band (M_r 38,000) on immunoblotting the BJ38 sample (Fig. 1, lane a). This band was not observed with control preimmune serum (Fig. 1, lane h). However, the same BJ38 sample yielded multiple bands when immunoblotted with anti-Brj antibody (Fig. 1, lane e). This latter antibody had been generated against heat-killed *B. japonicum* bacteria and had been shown to react with LPS and other bacterial components (6). The "ladder" pattern obtained when the BJ38 sample was immunoblotted with anti-Brj (Fig. 1, lane e) was similar to those obtained when LPS or CPS isolated from *B. japonicum* were immunoblotted with the same antibody (Fig. 1, lanes f and g). These results suggest that although the BJ38 sample was purified in terms of polypeptides, there was nonetheless a small amount of contamination by LPS and CPS.

For the purpose of the intended use of the anti-BJ38 antiserum as a reagent for immunolocalization, it was important to establish that anti-BJ38 reacted only with BJ38 and not with any contaminating polysaccharides. The results indicate that anti-BJ38 specifically reacted with BJ38, but did not react with either LPS or CPS fractions derived from *B. japonicum* (Fig. 1, lanes b and c). As an additional

Figure 1. Immunoblot analyses of BJ38, CPS and LPS isolated from *B. japonicum*. Lanes a, d,e,h, BJ38 sample (0.1 μg) isolated by 0.1 M Lac elution of *B. japonicum* cell extracts that had been fractionated on a Lac-Sepharose column; Lanes b,f,i, LPS (0.4 μg); Lanes c,g,j, CPS (0.4 μg). The samples were electrophoresed on 10% acrylamide gels, transferred to immobilon-P membrane and probed with anti-BJ38 (lanes a-c), monospecific anti-BJ38 (lane d), anti-Brj (lanes e-g), and preimmune serum (lanes h-j); all sera were used at 1:20 dilution. Alkaline-phosphatase conjugated goat anti-rabbit immunoglobulin antibody (1:1000) was used as the secondary antibody. Immunoreactive materials were then developed with nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate as substrates. The arrow on the left indicates the position of migration of a polypeptide of M_r 38,000, relative to known molecular weight markers.

a b c d e f g h i j

precaution, we affinity-purified the anti-BJ38 bound to the M_r 38,000 band on immunoblots. This affinity-purified monospecific antibody preparation yielded identical results as the original anti-BJ38 antiserum in immunoblotting (e.g. Fig.1, lane d) and in immunofluorescence experiments.

Immunofluorescence of BJ38 on *B. japonicum* Cell Surface

The availability of anti-BJ38 antibody allowed for the localization of BJ38 on the bacterial surface. When *B. japonicum* cells were labeled with anti-BJ38 antiserum, followed by FITC-conjugated goat anti-rabbit IgG, immunofluorescent staining of BJ38 was observed uniquely at one pole of the cell (Fig. 2a). The remainder of the cell surface was not labeled. A similar pattern of labeling was also observed with the monospecific affinity-purified anti-BJ38 antibody. Under our optimal conditions for labeling, about 70% of the bacterial population showed anti-BJ38 staining.

In contrast, when anti-Brj antibody was used to label the *B. japonicum* cells, immunofluorescence staining was observed evenly distributed throughout the entire cell surface (Fig. 2b). The preimmune antibody did not show any fluorescent labeling (Fig. 2c). These results indicate that BJ38 is located at one tip of the bacterium.

Ultrastructural Confirmation of Polar Localization of BJ38

To confirm the polar localization of BJ38 on *B. japonicum* at the ultrastructural level, *B. japonicum* cells were first incubated with anti-BJ38, followed by colloidal gold-labeled anti-rabbit antibody. BJ38 was found at one pole of the bacterial cell

Figure 2. Immunofluorescent labeling of *B. japonicum* cell surface by anti-BJ38, anti-Brj and preimmune sera. Cells were fixed on the activated coverslips, blocked in 5% BSA/YEG for 2 h and then incubated in (a) anti-BJ38, (b) anti-Brj, and (c) preimmune serum for 4 h (all at 1:20 dilution). After washing in YEG, the samples were incubated with FITC-conjugated goat anti-rabbit IgG antibody (1:50) for 1 h. The samples were washed in PBS and observed under a fluorescence microscope. PH, phase contrast; FL, fluorescence. Bar represents 3 μm.

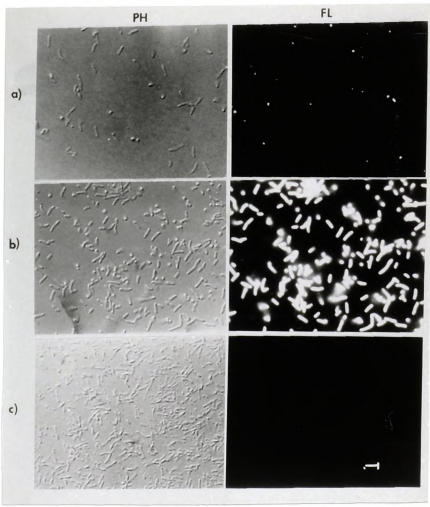
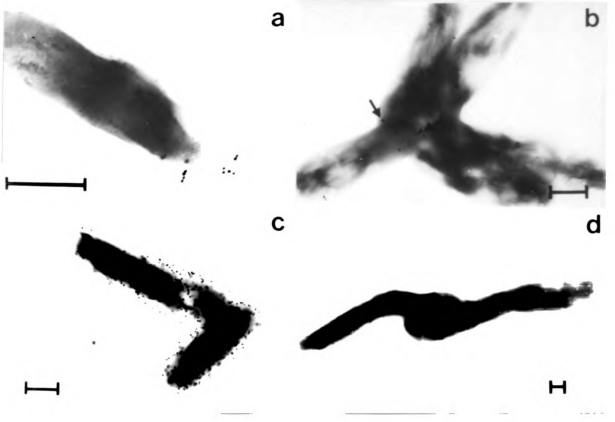

PH
FL
a)
b)
c)

Figure 3. Immunogold labeling of *B. japonicum* cell surface by anti-BJ38, anti-Brj and preimmune sera. a, cells from suspension culture; b-d, cells from star aggregates grown on a YEG agar plate. Suspension cells or star aggregates were fixed with glutaldehyde, blocked in 5% BSA/PBS for 1 h and then incubated in (a-b) anti-BJ38; (c) anti-Brj; and (d) preimmune serum for 4 h (all at 1:20 dilution). After washing in PBS, the samples were incubated with goat-anti-rabbit IgG antibody coupled with colloidal gold (1:5 dilution, particle size of 20 nm) for 1 h. The samples were washed in PBS and observed under an electron microscope. Bar represents 0.5 μm.


a
b
c
d

(Fig. 3a), as was seen with the immufluorescence data. Moreover, BJ38 appeared to be in a tuft-like mass, localized at a distance away from the outer membrane of the bacteria. A similar result was obtained by negative staining with phosphotungstic acid after immunogold labeling. The positions of the gold particles did not appear to be associated with any filamentous or pilus structure.

As expected, when anti-Brj antibody was used as the primary antibody, immunogold labeling was observed throughout the cell surface (Fig. 3c). Preimmune serum showed no labeling of the cell surface (Fig. 3d).

Identification of BJ38 at the Point of Contact

When *B. japonicum* was cultured on YEG agar plates, the bacteria tend to form aggregates (stars) by attaching to each other at one of their tips. Previous study had shown that this star formation can be inhibited by galactose and Lac (4). It was proposed that BJ38 might be involved in the star formation of *B. japonicum*. When these star-forming bacteria were used for BJ38 immunolocalization studies, immuno-fluorescence was observed at the focal point of cell-cell contact (Fig. 4a). This result suggests that BJ38 is present at the contact point where the bacteria interact with each other. This data is supported by the results of electron microscopic studies using immunogold labeling techniques (Fig. 3b).

When *B. japonicum* was allowed to attach to SB-1 cells or Lac-Sepharose beads, immunofluorescence analysis also indicates that BJ38 is localized at the point of contact (Fig. 4b,c). In contrast, there was no labeling of the other end of the

Figure 4. Immunofluorescent labeling of *B. japonicum* cells that were attached to a) other star-forming *B. japonicum*; b) SB-1 cells; and c) Lac-Sepharose bead. The *B. japonicum* were blocked in 5% BSA/YEG for 2 h and then labeled with anti-BJ38 antiserum (1:20 dilution) for 4 h. After washing in YEG, the samples were incubated for 1 h with FITC-conjugated goat-anti-rabbit IgG antibody (1:50). The samples were washed with PBS and then viewed under a fluorescence microscope. PH indicates phase contrast; FL, fluorescence. Bar represents 3 μm.

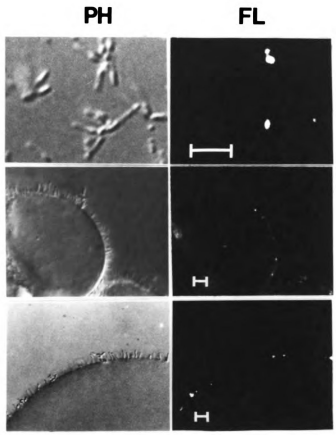
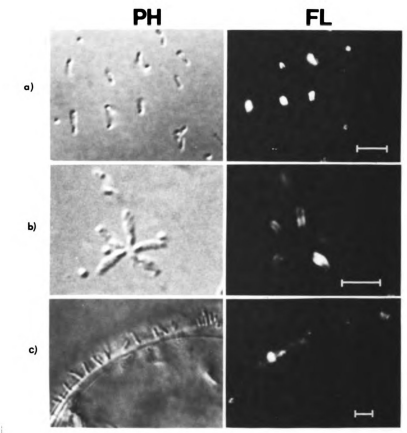

PH
FL
a)
b)
c)

Figure 5. FITC-SBA labeling of *B. japonicum* cells that were a) in suspension; b) in star-formation; c) attached to SB-1 cells. FITC-SBA (5 μg/ml) was incubated with the *B. japonicum* cells for 30 min. The cells were then washed with PBS and observed under a fluorescence microscope. PH represent phase contrast; FL, fluorescence. Bar represents 3 μm.


PH
FL
a)
b)
c)

bacterial cell. It should be noted that not all of the attached *B. japonicum* cells were labeled, possibly due to the low sensitivity of the immunofluorescence technique or hindered accessibility of the antibody. In any case, the results obtained with anti-BJ38 were distinctly different from the corresponding data using SBA.

When FITC-SBA was used to label the *B. japonicum* cells, a cap-like structure was observed covering a third of the bacterial cell (Fig. 5a). This was strikingly different from the point fluorescence observed with anti-BJ38 (Fig. 2a). When FITC-SBA was used to label the star-forming *B. japonicum* cells, FITC-SBA labeling was shown to be at the pole of the bacterium away from the attachment point (Fig. 5b). No fluorescent labeling was observed at the center of the stars, where *B. japonicum* cells attached to one another. Similarly, SBA labeled *B. japonicum* cells which were attached to SB-1 cells at the end of the bacterium, away from the point of contact (Fig. 5c). These data indicate that the receptor site for SBA binding is distinctly different from the site where BJ38 is localized.

DISCUSSION

The experiments reported in this paper were performed to determine the localization of BJ38 on the bacteria, using an antiserum directed specifically against the lectin. The results provide several key items of information. First, BJ38 is found at the cell surface. Second, BJ38 is localized at the pole of the bacterial cell which is involved in: a) homotypic adhesion to other star forming *B. japonicum*; b) heterotypic binding to SB-1 cells; and c) attachment to Lac-Sepharose beads. Since these binding processes occur in a polar fashion (4), the distribution of BJ38 has satisfied three topological requirements necessary for it to mediate bacterial adhesion. These are that BJ38 must be localized in a polar fashion, that it appears on the bacterial cell surface, and that the lectin must be found in the region of cell-cell contact.

The pole of BJ38 localization is distinct from that labeled by SBA. Consistent with our previous report (15), SBA labeled the pole of the bacteria away from which bacterial attachment occurred. In addition, SBA also labeled approximately a third of the cell surface. The latter result indicates that SBA-binding polysaccharides (12, 16, 17) were covering a third of the bacterial cell surface, forming a cap like structure. This is consistent with the observations of Tsien and coworkers (18, 19). They showed, by ruthenium red staining in transmission electron microscopy, that the capsular polysaccharides in *B. japonicum* were unevenly distributed on one half of the bacterium. They further showed that the internal structure of the bacterium can be divided into two distinct poles: the nucleoid portion containing chromosome and

cytoplasmic components and the reserve polymer half which contains glycogen and poly-beta-hydroxybutyrate granules. Ruthenium red staining indicates that SBA-binding polysaccharides are found in the nucleoid portion of the bacteria (19). In contrast, the granular portion is the half with which the bacterium participates in star formation. In the present study, we have shown that BJ38 and the SBA receptor reside at opposite ends of *B. japonicum*. The granular portion is thus the end at which BJ38 would be located.

Tsien and Schmidt (19) observed tuft-like structures in *B. japonicum* and termed them extracellular polar bodies. They contain fibrillar material of both polysaccharide and protein. It was suggested that these extracellular polar bodies mediate star formation. Indeed, our own ultrastructural localization of BJ38 in a tuft-like mass at a distance away from the bacterial outer membrane is consistent with this hypothesis and with the notion that the lectin plays a key role in the autoagglutination event.

On the other hand, our electron microscopic studies also indicate that BJ38 does not appear to be associated with any well-defined proteinaceous filaments. Such filamentous pili have been found in *R. lupini* (20), *R. meliloti, R. leguminosarum*, and *B. japonicum* (21) and have been proposed to mediate attachment (22). It has been reported that the isolated pili of *B. japonicum* consists of protein subunits with M_r 21,000 and M_r 18,000 (23). The subunit molecular weight of BJ38 makes it unlikely that it is a part of the isolated pilus structure. Moreover, our previous gel filtration data, in which the purified BJ38 protein chromatographed to a position corresponding

to M_r 38,000 (13), suggest that the lectin most likely does not form polymers leading to a filamentous structure.

Taken all together, the results detailed in this paper provide further evidence to support the proposed role of BJ38 in mediating the carbohydrate binding properties of *B. japonicum*. Not only is BJ38 saccharide specificity similar to that of *B. japonicum*, but its topological distribution is consistent with the polar binding activities of the bacterium. The importance of this latter observation is further demonstrated in studies with the N4 and N6 mutants (24). These mutants showed no detectable amounts of BJ38 on the bacterial cell surface and a decreased ability to nodulate soybean roots as compared to the wild-type *B. japonicum*. These results indicate that BJ38 may play an important role in the symbiotic infection process.

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CHAPTER III

CARBOHYDRATE BINDING ACTIVITIES OF *BRADYRHIZOBIUM JAPONICUM*

Effect of Lactose and Flavones on the Expression of the Lectin, BJ38

ABSTRACT

BJ38 is a galactose/lactose-specific lectin (M_r ~ 38,000) found at one pole of *Bradyrhizobium japonicum*. It has been implicated in mediating the adhesion of the bacteria to soybean roots, leading to the establishment of a nitrogen-fixing symbiosis. When the ligand lactose is added to cultures of the bacteria for at least one hour prior to harvesting the cells for BJ38 isolation, the yield of the protein was found to be elevated in a dose-dependent fashion. Half maximal stimulation was observed at ~ 50 μM; the effect was saturated at ~ 1 mM, where a 10-fold higher yield of BJ38 was obtained. Saccharides with a lower affinity for BJ38 than lactose yielded a correspondingly smaller induction effect when compared at a concentration of 1 mM. The higher level of BJ38 induced by lactose is also manifested by an elevated amount of BJ38 detectable at the cell surface and by a higher number of *B. japonicum* cells adsorbed onto soybean cells. Surprisingly, the induction of BJ38 expression seen with lactose was also observed with certain, but not all, flavonoids that induce the *nod* genes of the bacteria; genistein mimicked the induction observed with lactose, whereas luteolin failed to stimulate BJ38 production.

INTRODUCTION

In previous studies (1), we documented that *Bradyrhizobium japonicum* exhibits four saccharide-specific binding activities: (a) adsorption to Sepharose beads derivatized with lactose (Lac); (b) autoagglutination (star formation); (c) binding to cultured soybean (SB-1) cells; and (d) adhesion to soybean roots. In all four of these assays, galactose (Gal) inhibited the binding, but C-2 derivatives of the monosaccharide (e.g., N-acetyl-D-galactosamine, (GalNAc)) failed to yield the same effect. Mutants of *B. japonicum*, isolated on the basis of a defect in one binding activity (SB-1 cell binding), showed a concomitant loss in the other three binding capacities. These observations suggested that all of these carbohydrate-specific processes may be mediated by the same component and mechanism.

On the basis of these observations, we began to search for Gal/Lac-specific carbohydrate-binding proteins from *B. japonicum*. We reported the purification of one such lectin, designated BJ38 (M_r ~ 38,000), that bound Lac and Gal but showed much lower affinity toward GalNAc (2). A polyclonal antiserum generated against BJ38 was used in transmission electron microscopy and in confocal fluorescence microscopy to localize the lectin at the cell surface of the bacterium (3). More importantly, the lectin was found at only one pole of the cell, coincident with the attachment site for: (a) autoagglutination of other *B. japonicum* cells; (b) adhesion to soybean cells; and (c) adsorption to Lac-Sepharose beads. These results indicate that

the localization of BJ38 is consistent with a suggested role for this bacterial lectin in the polar binding of *B. japonicum* to other cells and surfaces.

In the course of these studies, we observed that the yield of BJ38 purified from *B. japonicum* was consistently higher when isolated from cultures containing Lac than from corresponding cultures without the disaccharide. Therefore, a systematic study was undertaken to document the requirements, specificity, and the consequences of this Lac effect. We report in the present communication the data derived from such a study, as well as the surprising observation that the Lac induction effect can also be mimicked by flavonoid compounds that are a part of the signaling components between the *Rhizobium* bacteria and their leguminous hosts.

MATERIALS AND METHODS

Bacterial Cultures and Isolation of BJ38

B. japonicum cells (R110d) were obtained from the laboratory of the late Dr. Barry Chelm (Michigan State University). The bacterial strain was maintained on agar plates containing yeast extract gluconate (YEG) as described before (3). Liquid cultures of *B. japonicum* were initiated by inoculating *B. japonicum* (3-day-old cells) from YEG agar plates into 50 ml YEG and culturing the bacteria on a gyratory shaker (100 rpm, 30°C) for two days. This culture was then inoculated into 2 liters of YEG. After two days, aliquots of *B. japonicum* (300 ml) were inoculated into 1.5 liters of YEG and the bacteria cultured for different lengths of time (see below).

Routinely, saccharides and flavonoids were added to this final liquid culture step to determine their effect on BJ38 expression. Lac was obtained from Kodak (Rochester, New York); mannose, GalNAc, genistein, apigenin, naringenin from Sigma (St. Louis, MO); and glucose from MCB (Cincinnati, OH). Dr. Franz De Bruijn (Michigan State University) kindly provided the luteolin.

For the isolation of BJ38, *B. japonicum* cells were harvested by centrifugation (11,000 g, 15 min) in a GS3 rotor and the bacteria were lysed by passage through a French Press (20,000 psi). The lysate was centrifuged at 27,000 g for 30 min and the supernatant fraction was precipitated by ammonium sulfate (65% saturation). The precipitate was centrifuged and resuspended in 3 ml phosphate-buffered saline (PBS) and dialyzed against PBS overnight at 4°C. The bacterial lectin was then isolated by

affinity chromatography on a Lac-Sepharose column (2). Lac-eluted fractions containing BJ38 were identified by SDS-PAGE (4) and silver staining (5). Samples were first concentrated by precipitation with 0.015% deoxycholate - 7.2% trichloro-acetic acid (6), washed with acetone, resuspended in sample buffer of the SDS-PAGE system, and subjected to electrophoresis. The gels were silver-stained and the intensities of the bands corresponding to BJ38 were quantitated on the basis of comparison with the staining intensities of known amounts of an arbitrary standard, carbonic anhydrase. Protein determination of cell extracts was performed by the method of Bradford *et al.* (7).

Use of [^{125}I] Anti-BJ38 to Quantitate Cell Surface Expression of BJ38

The generation and characterization of the anti-BJ38 antiserum used in this study have been reported previously (3). The immunoglobulin fraction of this antiserum was isolated by adsorption onto protein A-Sepharose (Sigma, St. Louis, MO) and was then labeled with ^{125}I by the chloramine T method (8). Na^{125}I (1 mCi) was added to 100 μl of antibody and chloramine T (1 mg/ml; 25 μl) was added to start the reaction. The reaction was stopped with the addition of 25 μl of sodium metabisulfite (1 mg/ml) in PBS. Free ^{125}I was removed by passage through an AG1X8 ion exchange column (Bio-Rad Laboratories, Richmond, CA). Radioactivity was determined in a γ-spectrometer counter (LKB Instruments, Inc., Rockville, MD).

For quantitation of cell surface BJ38, aliquots of *B. japonicum* cells (5 x 10^6) were first incubated in PBS containing 5% bovine serum albumin (BSA) for 1 hour at

4°C. ^{125}I-labeled anti-BJ38 (specific activity = 11.7 Ci/g) was added to the *B. japonicum* suspension. After 4 hours, individual samples were filtered through a membrane filter (pore size 0.22 μm, Millipore, Naperville, IL) that had been pre-soaked in 5% BSA/PBS. The membrane was washed three times in 0.1% BSA/PBS and the bound radioactivity determined.

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

SB-1 cells, originally derived from soybean roots (*Glycine max* (L.) Merr. cv. Mandarin) was kindly provided by G. Lark (Department of Biology, University of Utah, Salt Lake City, UT). Continuous cultures were maintained in 1B5C media by transferring 15 ml of culture (4-day-old) to 50 ml fresh 1B5C and culturing the suspension cultures at 27°C on a gyratory shaker in the dark.

For quantitation of bacterial binding to SB-1 cells, *B. japonicum* cells were washed three times in YEG to remove the saccharides that had been added to the YEG media. A binding assay described by Ho *et al.* (9) was then used. SB-1 cells (2-day-old, 0.5 ml of cell suspension (20% v/v)) were incubated with 10^9 *B. japonicum* in a 35 mm culture dish. After incubation for 0.5, 2 and 4 hours, the cells were transferred to 12 x 75 mm culture tubes and washed three times with PBS by centrifugation (460 g, 2 min) and resuspension.

Bacterial cells bound to the soybean host were quantitated by radioimmunoassay, using anti-Brj antibody (antibody generated against whole *B. japonicum* cell) (9). The cells were incubated with anti-Brj antibody (75 μg/ml containing 10^6 cpm [^{125}I]

anti-Brj (specific activity 2.32 Ci/g)) at 4°C. The samples were washed three times by centrifugation (460 g, 2 min) in 0.1% BSA/PBS and the bound radioactivity was quantitated to compare the relative amount of *B. japonicum* binding to SB-1 cells. In parallel, the conclusions derived from the quantitative assay were confirmed qualitatively by microscopic examination.

Fluorescence Microscopy

B. japonicum cells were attached to coverslips as described (3). The cells were blocked in 5% BSA/PBS for 2 hours, followed by incubation in either preimmune or anti-BJ38 antiserum (1:20 in 5% BSA/PBS). Following washing in PBS three times, fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit antibody (1:50 dilution; Boehringer Mannheim, Indianapolis, IN) in 5% BSA/PBS was added. After 1 hour incubation, the samples were washed three times with PBS. Stained cells were visualized with a fluorescence microscope (Zeiss D-7802 Obserkochen; excitation filter, 546 nm; chromatic beam splitter, 580 nm; barrier filter, 590 nm).

Glutamine Synthetase Assay

Glutamine synthetase activity was determined using the γ-glutamyl transferase (γ-GT) assay described by Bender *et al.* (10). Cell extract (μl amounts) was added to the γ-GT assay mixture consisting of 135 mM imidazole, 18 mM hydroxylamine, 0.27 mM $MnCl_2$, 25 mM potassium arsenate, 0.36 mM sodium ADP, pH 7.55. The

samples were incubated at 37°C for 5 min. The reaction was initiated by addition of L-glutamine (20 mM final concentration). Addition of "stop-mix" (0.2 M $FeCl_3.6H_2O$, 0.12 M trichloroacetic acid, and 0.25 M HCl) terminated the reaction. Precipitate was removed from the samples by centrifugation and the absorbance at 540 nm was determined. Using the value that 1 μmol of glutamyl hydroxamate gives 0.532 units of absorbance at 540 nm as determined by Bender *et al.* (10), the specific activity of glutamine synthetase was calculated and expressed as μmol γ-glutamylhydroxamate produced per minute per mg cell extract.

RESULTS

Lactose Induction of BJ38 Expression in *B. japonicum*

In the protocol for the isolation of BJ38, liquid cultures of *B. japonicum* were initiated by inoculating the bacteria maintained on agar plates in YEG medium. This initial liquid culture (50 ml) was expanded twice (for details, see Material and Methods). The final liquid culture (~ 2 liters) reached late logarithmic phase in ~ 27 h, at which time the cells were harvested for lectin purification. Addition of Lac to the final liquid culture resulted in a 10-fold increase in the yield of BJ38, when compared to the corresponding yield from cultures without Lac (Fig. 1). The effect of Lac was dose-dependent, with saturation at ~ 1 mM. Half maximal effect was observed at ~ 50 μM.

Three sets of experiments were performed to determine the length of time required to observe the Lac effect. First, Lac was added to a final concentration of 1 mM at various times during the final liquid culture and all the cultures were harvested at the same time (i.e. at the end of the 30-h period of the final liquid culture). In this scheme, the cell density and growth phase of the bacteria at the time of harvest were kept constant, while the initial time (cell density and growth phase of bacteria) of exposure to Lac, as well as the length of exposure to Lac, were allowed to vary. The results showed that Lac added at early time points during the final liquid culture yielded the greatest effect (Fig. 2A). Thus, addition of Lac at 2 h and 8 h of the final

Figure 1. Dose-response curve of the effect of Lac on the production of BJ38. *B. japonicum* cells were cultured for 27 h in YEG media that had been supplemented with different concentrations of Lac. The bacterial cells were lysed by French pressing and the cell extracts were fractionated over a Lac-Sepharose column. BJ38 was eluted with 0.1 M Lac. The amount of lectin was quantitated by comparing the intensities of the silver-stained polypeptide bands (inset) with the staining intensities of known amounts of carbonic anhydrase, and is presented in the figure as ng of BJ38 isolated per mg of cell extract.

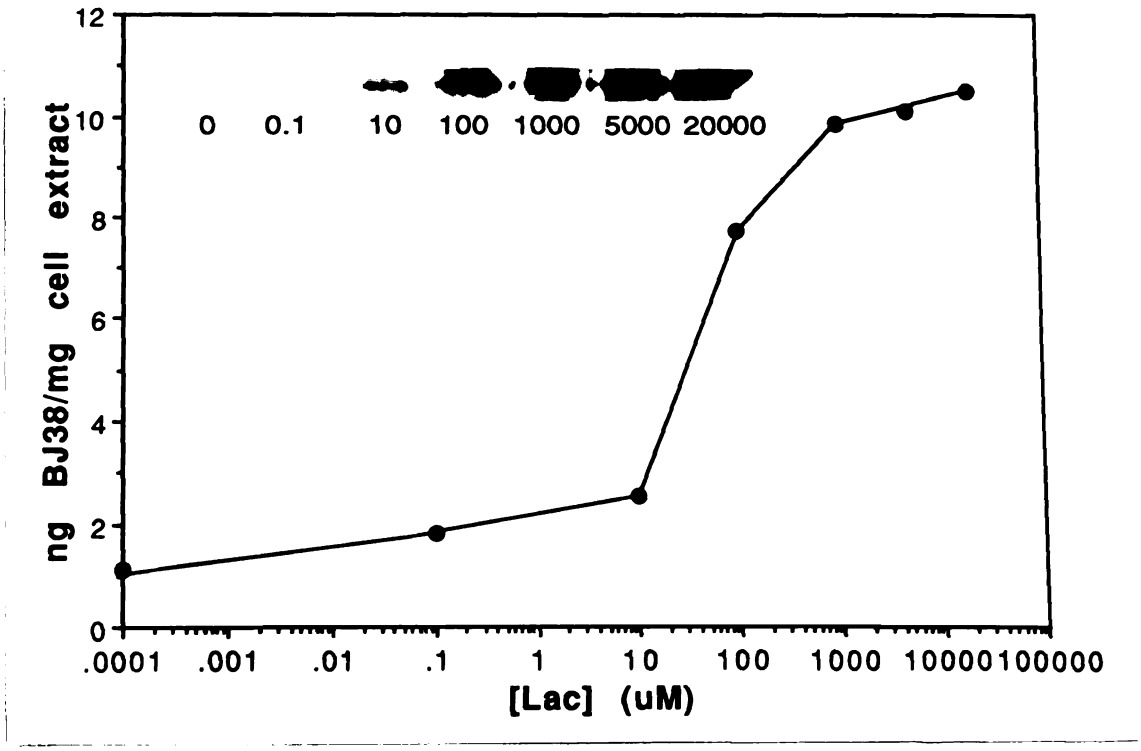
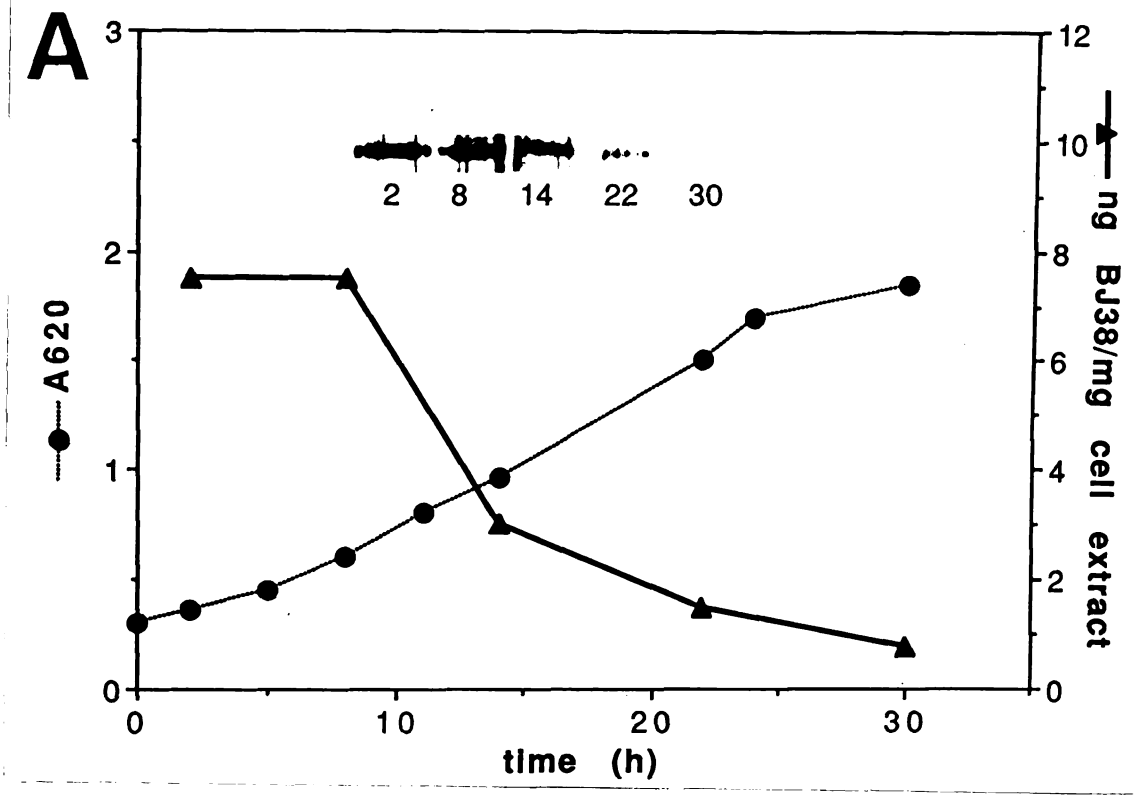
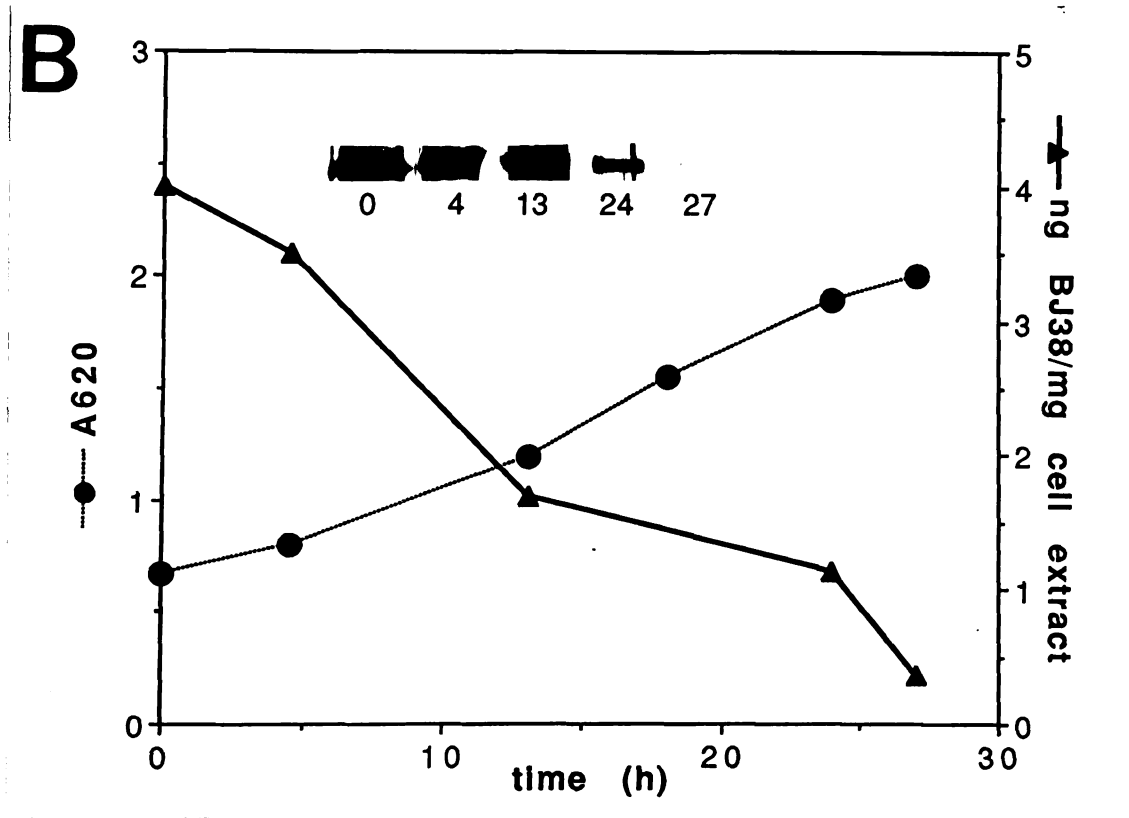

12
10
8
6
4
2
0
ng BJ38/mg cell extract
0
0.1
10
100
1000
5000
20000
.0001
.001
.01
.1
1
10
100
1000
10000
100000
[Lac] (uM)

Figure 2. The effect of the growth phase of *B. japonicum* cells on the Lac induction of BJ38 expression. Lac was added to a concentration of 1 mM at various times during the final liquid culture (300 ml of a 2 liter *B. japonicum* culture passaged into 1.5 liter YEG). In A, all cultures were harvested at the same time (~ 30 h after initiation of the final liquid culture). In B, the cultures were harvested at different times (in each case, 4 h after the addition of Lac). The cell density of the sample at the time of harvest is presented as absorbance at 620 nm (A620 ●). The amount of BJ38 isolated is expressed as ng BJ38/mg cell extract (▲). The inset shows the intensities of the silver-stained bands of the isolated BJ38 normalized to total protein added. These experiments were repeated three times, with essentially the same results.

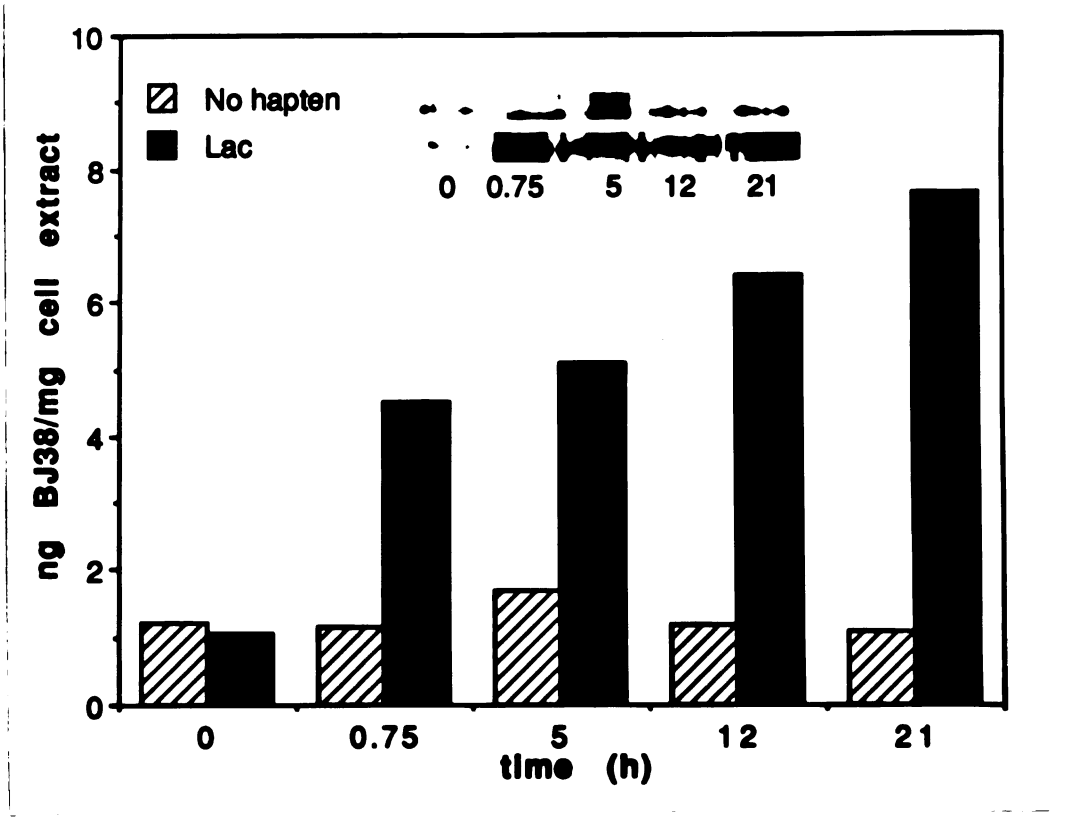

A
2 8 14 22 30
A620
ng BJ38/mg cell extract
time (h)
0
10
20
30


B
0 4 13 24 27
A620
ng BJ38/mg cell extract
time (h)
0
10
20
30

liquid culture resulted in an approximately 8 times higher yield of BJ38 than if Lac was added immediately before the harvest. In such an experimental scheme, we could not distinguish whether the observed differences were due to a difference in the length of exposure of the *B. japonicum* cells to Lac or to the time (cell density and/or growth phase) of the initial exposure.

Therefore, the second experimental scheme tested the effect of Lac addition and harvest at various times throughout the final liquid culture period of 30 hours, keeping the length of exposure constant at 4 h. The results showed that exposure to Lac early during the final liquid culture period yielded the highest amount of BJ38, with a monotonic decrease in the yield with later times of exposure (Fig. 2B). It should be noted that the yield of BJ38 is expressed as ng of BJ38 isolated per mg of cell extract so that the value is normalized to the total cell number used in the sample. Thus, the results suggest that the cell density and/or growth phase of the bacteria was critical at the time of Lac addition. In this experiment, however, the density (and the growth phase) of the *B. japonicum* cells at the time of harvest for BJ38 isolation was different for each sample. Therefore, a third experimental scheme was devised to eliminate this variable. Lac was added at the initiation of the final liquid culture period and the cells were harvested after different lengths of exposure to the saccharide. The results showed that the control samples (*B. japonicum* cells without any Lac) yielded "baseline" levels of BJ38, while Lac-treated cells yielded increasing amounts of BJ38 with longer exposures to the saccharide (Fig. 3). Exposures as short as three-quarters of an hour were sufficient for appreciable enhancement of the yield of BJ38. The high yield of BJ38 in the sample that received 21 h of exposure to Lac

Figure 3. The effect of time of exposure of *B. japonicum* cells to Lac on the induction of BJ38 expression. Lac was added to a concentration of 1 mM at the initiation of the final liquid culture (300 ml of a 2 liter *B. japonicum* culture passaged into 1.5 liter YEG) and the cultures were grown for the indicated lengths of time before harvesting the cells for BJ38 isolation. In parallel, final liquid cultures containing no saccharide were grown for the same lengths of time prior to harvesting for BJ38 isolation. The amount of BJ38 isolated is expressed as ng BJ38/mg cell extract. The inset shows the intensities of the silver-stained bands of the isolated BJ38 normalized to total protein content. This experiment was repeated three times with similar results.


10
8
6
4
2
0
ng BJ38/mg cell extract
No hapten
Lac
0 0.75 5 12 21
0
0.75
5
12
21
time (h)

was important because this showed that the high cell density and the stationary phase of the bacteria at the time of harvest did not impair the Lac effect.

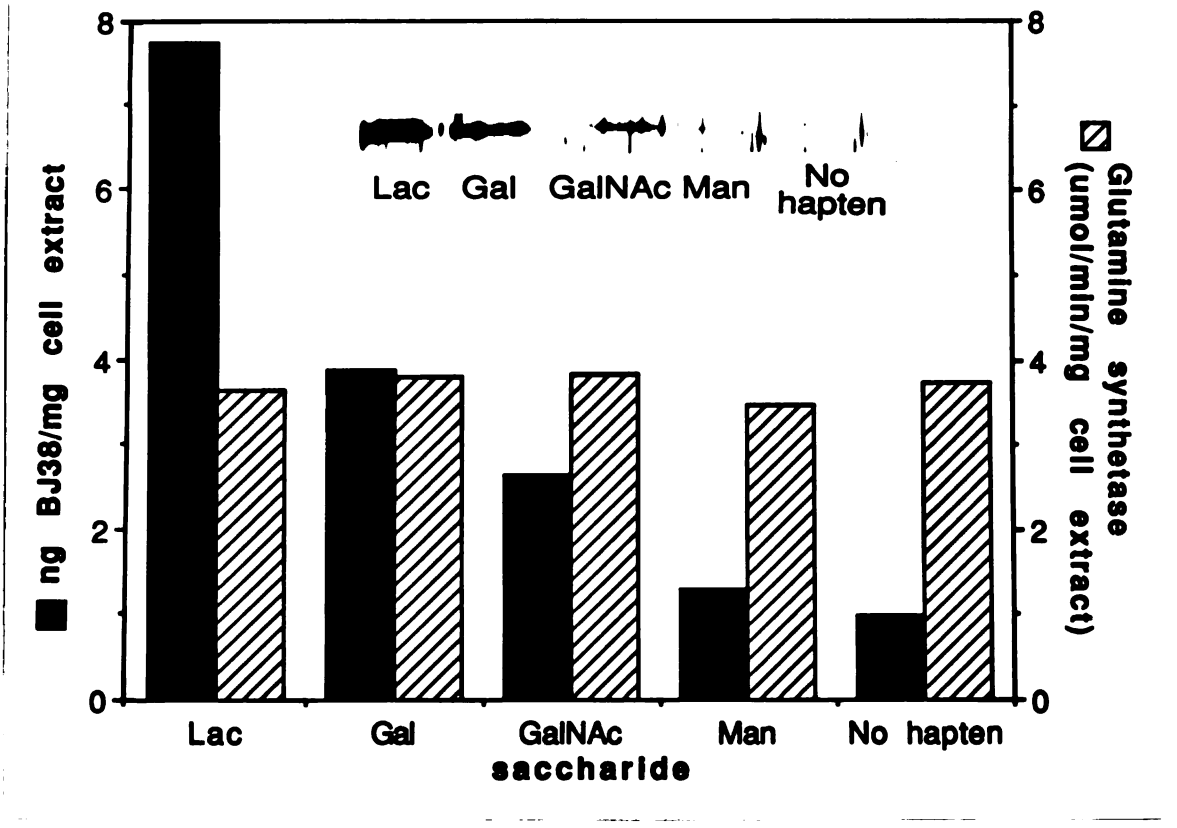
Specificity of the Lactose Induction Effect

The specificity of the Lac effect was tested in terms of two key questions: (a) can other saccharides mimic the effect of Lac? and (b) does Lac affect the expression of other *B. japonicum* proteins, besides BJ38? Glutamine synthetase was chosen as an irrelevant protein control, to be compared to BJ38. Thus, aliquots of the same *B. japonicum* culture were inoculated into the final liquid YEG step that contained either no hapten (control) or various saccharides (1 mM). For each of these final liquid cultures, the amount of BJ38 isolated and the specific activity of glutamine synthetase were quantitated (Fig. 4). Neither Lac, nor any of the other saccharides, altered the level of activity of glutamine synthetase. On the other hand, the stimulatory effect of Lac on the yield of BJ38 was much more pronounced than the other saccharides. The 8-fold induction due to Lac should be compared to the 4-fold induction seen with Gal and the 2.5-fold effect due to GalNAc. This relative order of the ability to induce BJ38 expression is similar to the relative binding affinity of BJ38 for the three saccharides (2). Finally, Man, which does not bind to BJ38 (2), had little or no effect on BJ38 expression.

Effect of Lactose Induction on Cell Surface Expression of BJ38

Thus far, the expression of BJ38 has been assayed at the level of amount of the lectin that can be purified by Lac-Sepharose affinity chromatography. The

Figure 4. Saccharide specificity in the induction of BJ38. Final liquid cultures of *B. japonicum* were incubated for 10 h in the presence of 1 mM concentrations of Lac, Gal, GalNAc, and Man, or without any saccharide. The amount of BJ38 isolated (inset) was quantitated and is expressed as ng BJ38/mg cell extract. In parallel, the specific activity of glutamine synthetase in the cell extracts was also determined (see Materials and Methods). This experiment was reproduced three times.


8
6
4
2
0
ng BJ38/mg cell extract
Lac
Gal
GalNAc
Man
No hapten
Lac
Gal
GalNAc
Man
No hapten
saccharide
Glutamine synthetase (umol/min/mg cell extract)

question is, therefore, raised whether the induction of BJ38 expression by Lac is also manifested at the level of increased amounts of BJ38 at the cell surface. BJ38 at the cell surface was detected in two assays, using a polyclonal rabbit antiserum specific for the lectin (3). First, rabbit anti-BJ38 was labeled with ^{125}I and the amount of antibody required to saturate the BJ38 found on the cell surface of *B. japonicum* was determined by quantitating the radioactivity bound as a function of antibody added (Fig. 5, inset). Using a saturating dose of ^{125}I-labeled anti-BJ38 ($\sim$ 44 ng), the level of BJ38 expressed on the cell surface was compared for *B. japonicum* cells derived from final liquid cultures containing various saccharides (1 mM) or containing no hapten (control). The presence of Lac in the final liquid culture greatly increased the amount of BJ38 at the cell surface, accessible to binding by ^{125}I-labeled anti-BJ38 (Fig. 5). The other saccharides had little or minimal effects. This includes GalNAc and Gal, which yielded 2.5- to 4-fold induction effects when the expression of BJ38 was assayed at the level of isolation of bulk amounts of the lectin.

Second, immunofluorescence staining with rabbit anti-BJ38 was used to determine the fraction of *B. japonicum* expressing BJ38 at the cell surface. Approximately 40% of the bacteria derived from final liquid culture containing Lac (1 mM) stained with anti-BJ38 (Fig. 6a). In contrast, bacteria derived from final liquid cultures that contained no hapten or a control saccharide (1 mM glucose) yielded 10% immunofluorescent cells (Fig. 6b and 6c). Thus, assaying at the level of antibody-accessible BJ38, the induction effect of Lac is manifested mainly as an increase in the percent of cells exhibiting cell surface exposed lectin.

Figure 5. Quantitation of the effect of saccharides on the cell surface expression of BJ38. *B. japonicum* cells were cultured in the presence of Lac, Gal, GalNAc, Man, and Glc (all at 1 mM), or without any saccharide. Bacteria from each treatment (5 x 10^6 cells) were then blocked with 5% BSA/PBS for 1 h and then incubated for 4 h with 44 ng of [^{125}I]anti-BJ38. The bacteria were then filtered through a millipore filter (pore size 0.22 μm) and the membrane washed three times in PBS. The amount of radioactivity retained on the membrane was then determined. The data represent means $\pm$ standard deviations of triplicate determinations. Inset: Determination of the amount of anti-BJ38 needed to saturate the BJ38 found on the cell surface of *B. japonicum*. *B. japonicum* (5 x 10^6) were incubated with various amounts of [^{125}I]anti-BJ38 for 4 h. *B. japonicum* was then filtered through a millipore filter and the amount of bound radioactivity determined.

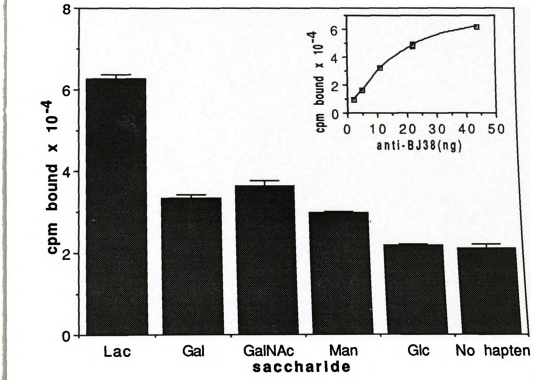
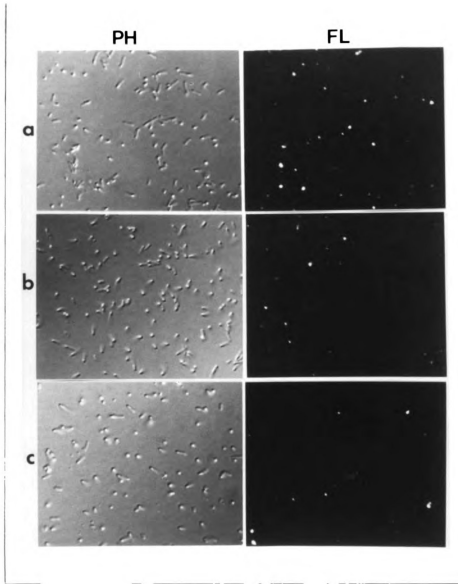

cpm bound x 10⁻⁴
8
6
4
2
0
Lac
Gal
GalNAc
Man
Glc
No hapten
saccharide
cpm bound x 10⁻⁴
6
4
2
0
0 10 20 30 40 50
anti-BJ38(ng)

Figure 6. The effect of saccharides on the expression of BJ38 at the cell surface of *B. japonicum* as detected by immunofluorescence staining with anti-BJ38. Bacteria cells were cultured in the presence of 1 mM concentrations of (a) Lac; (b) Glc; and (c) no hapten for 24 h. The cells were then stained with anti-BJ38 as described in the Materials and Methods. PH, phase contrast; FL, fluorescence.


PH
FL
a
b
c

Effect of Lactose Induction on the Adhesive Properties of *B. japonicum*

Since Lac induction of BJ38 expression is manifested not only at the level of total BJ38 isolatable but also in terms of the fraction of cells exhibiting BJ38 detectable at the cell surface, the question is raised whether the capacity of *B. japonicum* to bind to soybean cells is also increased upon Lac induction. Bacteria derived from Lac-containing final liquid cultures indeed showed a high rate of adhesion to cultured soybean SB-1 cells (Fig. 7). Within half an hour following co-culture of SB-1 cells with *B. japonicum*, bacteria derived from Lac-induced cultures showed many cells attached to the soybean cells, compared to the corresponding co-culture using uninduced bacteria.

We had previously developed a radioimmunoassay, using an antiserum raised against whole *B. japonicum* cells (anti-Brj), to quantitate the number of bacteria bound on soybean cells (9). The differences in the number of *B. japonicum* attached to SB-1 cells, qualitatively represented in the photomicrographs of Figure 7, are corroborated by the quantitative data (Fig. 8). There was about a 4-fold increase in the number of attached cells, comparing Lac-induced versus control samples, half an hour after co-culture. This difference decreased to about 2-fold upon longer term culture.

Figure 7. The effect of inclusion of Lac in the final liquid culture of *B. japonicum* on the subsequent binding of the bacteria to SB-1 soybean cells as observed by light microscopy. *B. japonicum* (10^9 cells) derived from final liquid cultures with (YEG + Lac) and without (YEG) saccharide were incubated with 0.5 ml cell suspension (20% v/v) of SB-1 cells as described in Materials and Methods. Light micrographs were taken at (a) 0.5, (b) 2, and (c) 4 h after *B. japonicum* was added to the SB-1 cells.

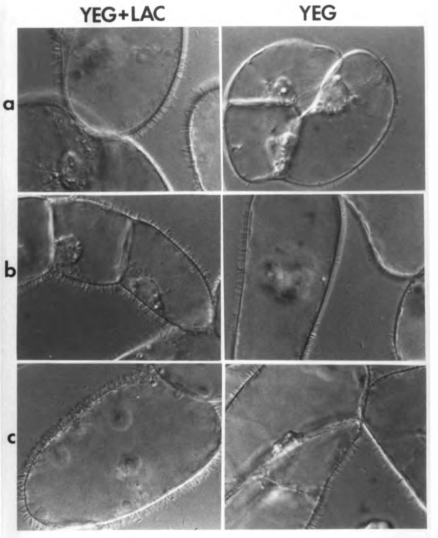

YEG+LAC
YEG
a
b
c

Figure 8. The effect of inclusion of Lac in the final liquid culture of *B. japonicum* on the subsequent binding of the bacteria to SB-1 soybean cells as quantitated by a radioimmunoassay. The experiment was carried out as in Figure 7 and the number of bacteria binding to the SB-1 cells was quantitated using [^{125}I]anti-Brj as detailed in Materials and Methods. The data represent means ± standard deviations of triplicate determinations.

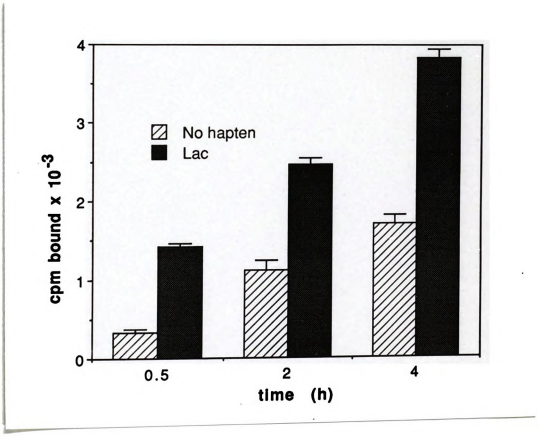
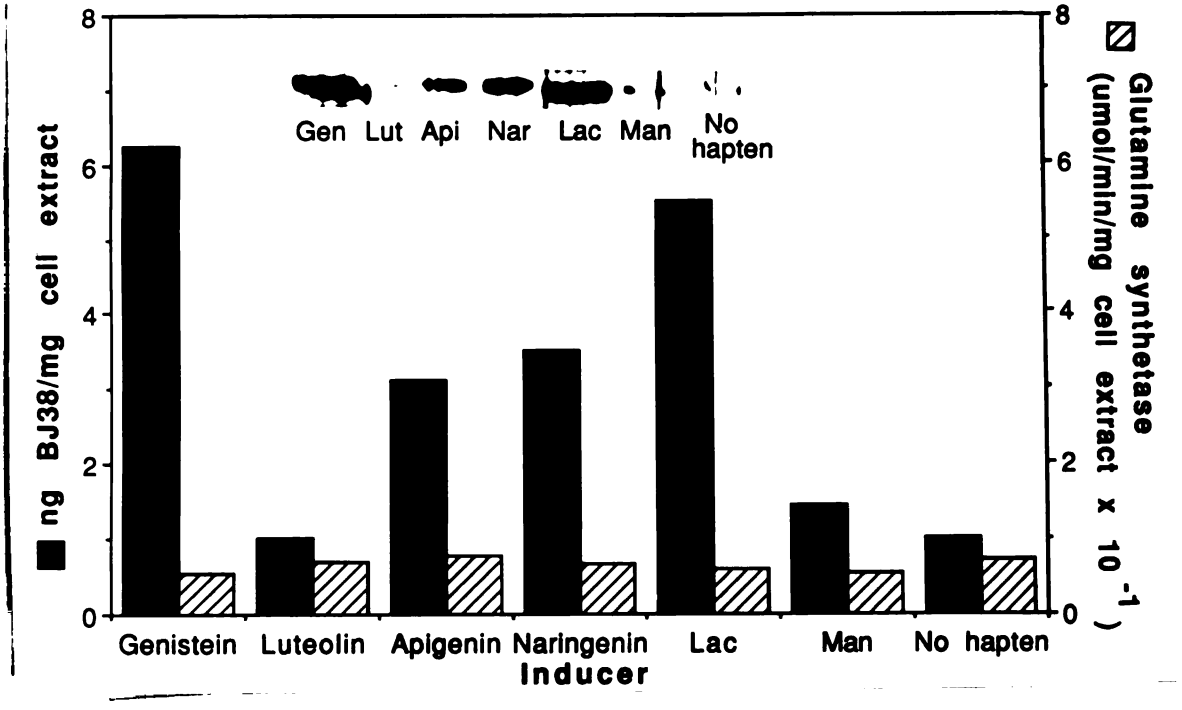

4
3
2
1
0
No hapten
Lac
cpm bound x 10-3
0.5
2
4
time (h)

Figure 9. Comparison of the effect of flavones and saccharides on the induction of BJ38 expression in *B. japonicum* cells. Final liquid cultures of *B. japonicum* were incubated for 20 h in the presence of flavones (2 μM) or saccharides (1 mM) or with no addition. The amount of BJ38 isolated (inset) was quantitated and is expressed as ng BJ38/mg cell extract. In parallel, the specific activity of glutamine synthetase in the cell extracts was also determined. This experiment was repeated three times with similar results.


Gen Lut Api Nar Lac Man No hapten
ng BJ38/mg cell extract
Glutamine synthetase (umol/min/mg cell extract x 10 -1)
0
2
4
6
8
Genistein
Luteolin
Apigenin
Naringenin
Lac
Man
No hapten
Inducer

Induction of BJ38 Expression by Flavones and Derivatives

Using the same protocol for Lac induction, we have also tested the effect of several flavonoid compounds implicated in the induction of the *nod* genes that are required for early host responses in the *Rhizobium*-host plant signalling (11-15). Thus, the flavonoids (2 μM) were included in the final liquid culture for 20 h prior to harvesting the bacterial cells for BJ38 isolation. The isoflavone genistein yielded a strong induction effect on BJ38 expression, comparable to that seen with the "positive control," Lac (Fig. 9). In contrast, the flavone luteolin failed to yield any effect; the amount of BJ38 isolated corresponded to those of "negative controls," Man and no hapten inducer. Finally, the flavone apigenin and the flavonone naringenin resulted in intermediate levels of induction of BJ38 expression.

DISCUSSION

The key findings of this study include: (a) Carbohydrate ligands of the lectin BJ38 can regulate the level of the protein; (b) This is manifested both in terms of the amount of protein that can be isolated on the basis of its carbohydrate-binding activity and in terms of cell surface exposed protein accessible to binding by a specific antibody; (c) Among the saccharides tested, Lac was the best inducer, with a half maximal effect observed at $\sim$ 50 μM and saturation at $\sim$ 1 mM. When tested at 1 mM, the order of potency in stimulating the expression of BJ38 was Lac > Gal > GalNAc.

There does not appear to be a direct correspondence between the amount of BJ38 isolated by affinity chromatography and the amount of BJ38 detected at the cell surface. Lac induced an 8- to 10-fold increase in the yield of BJ38, but the increase in the percent of cells with BJ38 detectable at the cell surface is only about 4-fold. Moreover, the increase in BJ38 assayed by these methods translates into a 2-fold increase in the number of cells adhering to soybean cells. These results raise the question whether the increase in BJ38 is due to an elevated expression of the lectin in cells that already contain the protein or may represent a recruitment of a new subpopulation of *B. japonicum* cells that do not exhibit BJ38 in the absence of the carbohydrate ligand. It is not known how this subpopulation of bacteria might be different from those *B. japonicum* cells expressing the protein.

At the molecular level, it is not known whether the Lac induction effect occurs at the transcriptional or post-transcriptional levels. For example, it is possible that ligand binding merely protects or sequesters the lectin from proteolytic degradation, thus resulting in high levels of the lectin. It seems more likely, however, that the induction effect is a result of transcriptional regulation of the BJ38 gene. Transcriptional regulation has been observed in the control of several saccharide binding proteins which mediate the transport of maltose, arabinose, xylose and lactose into bacteria (16-19). In these systems, the genes encoding the binding proteins are located in operons that are regulated by proteins which either activate or repress transcription of the operon. The transcription of these genes are regulated by the haptens of their respective gene products. In the case of BJ38, Lac is not the only saccharide inducer. Moreover, the potency of various saccharides in stimulating BJ38 expression follows the same order of affinities as between the lectin and its carbohydrate ligands. For example, we had previously quantitated that the binding of the disaccharide Lac was 13-fold higher than that of Gal, which in turn was about 18-fold higher than that of GalNAc (2). Consistent with this relative order of affinities, Lac induced an 8- to 10-fold increase in BJ38, while the effects of Gal and GalNAc were 4- and 2.5-fold, respectively. This raises the distinct possibility that it is BJ38 itself that is binding to the promoter region and regulating the transcription of its own gene. For example, binding of carbohydrate ligands may preclude BJ38 from repressing the transcription of its own gene, possibly due to a ligand-induced conformational change.

To our surprise, we found that several flavonoid compounds known to regulate the *nod* genes of *Rhizobium* (11-15) also induced BJ38 expression. In accord with previous reports (11,20), genistein, which was most potent in inducing *nodABC* expression, was also the most potent inducer of BJ38. Similarly, the flavone luteolin had negligible effects with respect to both *nodABC* and BJ38 induction and apigenin yielded induction levels intermediate between genistein and luteolin in both assays. Naringenin also induced BJ38, at a level below that of genistein but comparable to apigenin; this flavanone had no effect on *nod*ABC induction in *B. japonicum* cells (11,20).

These results raise the possibility that the BJ38 gene, demonstrated to be saccharide-sensitive, is also controlled by NodD, which mediates the activation of other *nod* genes (21,22). This scheme suggests that BJ38 may belong to the family of *nod* genes necessary for successful infection of the host plant. Such a possibility is intriguing in light of the fact that sequence analyses of known *nod* genes suggest that the *nodABC, nodH, nodL, nodM* and *nodZ* gene products are capable of binding carbohydrate (22-25). These carbohydrate binding proteins function as enzymes proposed to be involved in the synthesis of low molecular weight lipooligosaccharide compounds such as the *nod* factors essential for successful nodulation. In our present case, however, BJ38 would be a carbohydrate binding product of a *nod* gene whose function is to mediate the attachment of *B. japonicum* to soybean roots (1). This present scheme, however, does not preclude the possibility that flavonoids may stimulate BJ38 transcription through bacterial response pathways other than *nodD*.

For instance, studies by Parniske *et al.* (26) have also demonstrated isoflavonoid-induced resistance of *B. japonicum* to the phytoalexin glyceollin. This resistance has been demonstrated in *nodD$_1$D$_2$YABC* deletion mutants, suggesting alternative sites of genistein recognition.

At the functional level, the reasons for such a dual control of BJ38 expression can only be speculated at this point. Lac and Gal could function to enhance *B. japonicum* attachment to soybean roots. Lac and Gal are major constituents of the plant cell wall and may serve as a plant signal to enhance bacterial attachment to the soybean root through elevated levels of BJ38. Our present observation that an increase in *B. japonicum* binding to soybean roots is paralleled by an increase in the levels of BJ38 provide correlative evidence that this may indeed be so. A similar role for saccharide mediated induction has been proposed for *Agrobacterium tumefaciens.* Ankenbauer and Nester have reported that ChvE, a periplasmic sugar binding protein mediates the induction of the *vir* genes in *A. tumefaciens* (27, 28), leading to the infection of the host plant. As most of the sugars able to bind to ChvE and induce the *vir* genes are constituents of plant cell wall polysaccharides, it was postulated that these compounds served to signal the bacterium of the presence of the host plant and, therefore, activate the bacterium for infection. In a similar fashion, soybean wall constituents, specifically Lac and Gal, may serve as signals to *B. japonicum* and activate that bacterial strain's attachment to soybean roots.

Flavonoids could also function to enhance *B. japonicum* attachment to soybean roots. In previous studies (29), we had documented that both BJ38 and *B. japonicum*

bind preferentially to the emergent root hair zone of the soybean root. This preferential binding has been attributed to recognition of specific carbohydrate structures on the soybean root by BJ38. In studies on the spatial distribution of flavones capable of inducing *nod* gene expression along the plant root surface, the emergent root hair zone has also been shown to be the zone of maximum induction of bacterial *nod* genes by flavonoids (30-33). Given these observations and the results documented in the present study, induction of BJ38 expression by flavonoids would lead to an enhanced binding of *B. japonicum* to soybean roots and, in particular, to the emergent root hair zone.

ACKNOWLEGEMENTS

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CHAPTER IV

CARBOHYDRATE BINDING ACTIVITIES

OF *BRADYRHIZOBIUM JAPONICUM*

Analysis of the effects of saccharides and flavones

on the expression of BJ38 at the mRNA level.

FOOTNOTE

[1]The generation of the PCR1 probe and Southern blot analyses were performed by Dr. Siu-Cheong Ho.

ABSTRACT

BJ38 is a galactose/lactose-specific binding protein implicated in mediating the adhesion of *Bradyrhizobium japonicum* to soybean roots. In previous studies, we had demonstrated that BJ38 expression, assayed at the polypeptide level, was increased when *B. japonicum* cells were cultured in the presence of either lactose or the flavone genistein. In the present study, we have compared the effect of these inducers on the expression of BJ38 mRNA. Treatment of *B. japonicum* cells with saccharides and flavones resulted in enhanced BJ38 mRNA expression. Lactose yielded the greatest effect on BJ38 expression, followed by galactose; mannose had little effect on BJ38 mRNA levels. Of the flavones tested, induction of BJ38 mRNA was greatest with genistein, followed by apigenin. Luteolin showed little increase in BJ38 mRNA levels. Interestingly, a similar order of efficacy for induction by saccharides and flavones was also observed for the expression of the *nodD*$_1$ gene in *B. japonicum*.

INTRODUCTION

The rhizobium-legume interaction leads to the invasion of the roots of the host plant and the formation of a nitrogen-fixing symbiosis. A key step leading to successful infection and nodulation involves the attachment of bacteria to the host root. In the *Bradyrhizobium japonicum*-soybean interaction, the attachment of bacteria to the host-legume is galactose (Gal)-specific (1-3), suggesting the involvement of a carbohydrate-binding protein. Consistent with this observation, we have described the isolation of a carbohydrate binding protein, BJ38, from *B. japonicum*. BJ38 exhibits a saccharide specificity similar to that of bacterial adhesion to soybean roots (4). In addition, the lectin has also been immunolocalized at the attachment site of bacterial attachment to soybean cells (5). Purified BJ38 binds to soybean roots at sites coincident with *B. japonicum* attachment: namely, at the emergent root hair zones (6). These regions had previously been demonstrated by Bauer and coworkers (7, 8) to be the most susceptible to initiation of infection by *B. japonicum*. More recently, we have reported that the expression of BJ38 can be induced by both lactose (Lac) and the isoflavonoid genistein (9). As genistein is a potent inducer of the *nod* genes of *B. japonicum,* this latter result suggests the possibility that BJ38 may be a member of the *nod* gene family.

The *nod* genes comprise a set of key bacterial elements in the infection process and are transcribed in response to specific flavonoid compounds secreted from the host-plant (10-12). This requires the presence of the *nodD* gene product (13-15),

which, in association with the appropriate flavonoids, binds to the *nod* box promoter sequence preceeding the *nod* genes and activates the transcription of these genes (16, 17). In *B. japonicum,* two copies of *nodD*, $nodD_1$ and $nodD_2$, have been identified (18). Of these two *nodD* genes, only $nodD_1$ is both inducible by isoflavonoids and necessary for *nod* gene induction. In this present study, we document results which characterize the effect of saccharides and flavonoid compounds on the expression of BJ38 mRNA . Simultaneously, we also monitored the effects of these compounds on $nodD_1$ mRNA expression.

MATERIALS AND METHODS

Cell Cultures

B. japonicum cells (R110d) were maintained on YEG (yeast-extract-gluconate) agar plates for 3 days as described previously (9). The bacterial cells were then inoculated into 50 ml YEG and cultured in a gyratory shaker for 2 days. This culture was then transfered to 2 liter of YEG and grown for 2 more days. Aliquots of 300 ml of the 2 liter bacterial cultures were then transferred to 1.5 liter YEG to obtain the final liquid culture step. On the initiation of the final liquid culture, saccharides and flavones were added to a final concentration of 1 mM, and 2 μM, respectively and the bacteria cells were cultured for 10 h prior to analysis. Lac was obtained from Kodak (Rochester, New York); mannose, genistein, apigenin, naringenin from Sigma (St. Louis, MO). Luteolin was kindly provided by Dr. Franz De Bruijn (Michigan State University).

The *R. meliloti* 1021 and *R. leguminosarum* bv. *trifolii* (*R. trifolii)* ANU843 strains were kindly provided by Dr. Rawle Hollingsworth (Michigan State University) and maintained on agar plates containing Minimum Bergensens Media III (MBM) (19). Liquid cultures of these strains were initiated by inoculation of the bacterial cells (2 day old, MBM agar plates) into 50 ml MBM. The bacterial cells were grown for 12 h at 30°C before being transfered to 1 liter of MBM. This final 1 liter culture was then allowed to grow for 10 h.

Determination of the Partial Amino Acid Sequence of BJ38

Purified BJ38 (12 μg) was digested with *Staphylococcus aureus* V-8 protease (Miles Laboratories, Naperville, IL) as described by Cleveland et al. (20). The digestion was carried out with 0.6 μg of enzyme for 0.5 h. The peptides generated were subjected to SDS-PAGE electrophoresis (21), transfered to a problot membrane (Applied Biosystems, Foster City, CA) and the digestion products revealed by Coomassie blue staining. Amino acid sequencing was performed by the Edman degradation method (22) on a Model 477A Gas Phase Sequencer (Macromolecular Structure Facility, Michigan State University).

Polymerase Chain Reaction (PCR) Amplification and Nucleotide Sequence of PCR products

Using the partial amino acid sequences obtained for BJ38, degenerate oligonucleotide primers were synthesized based on the codon usage in *B. japonicum.* PCR amplification was peformed, with *B. japonicum* genomic DNA as a template, on an Automated DNA Thermal Cycler 9600 (Perkin Elmer Cetus, Norwalk, CT) using the protocol described by Saiki (23). The procedure consists of a denaturing step (94°C, 2 min) followed by 35 amplification cycles of denaturation (94°C, 1 min), annealing (55°C, 1 min) and extension (72°C, 2 min). For BJ38 amplification, 5 additional cycles [denaturation (94°C, 1 min), annealing (37°C, 5 min) and extension (72°C, 2 min)] were included prior to the 35 amplification cycles. Following amplification, further extension at 72°C (5 min) was allowed before cooling to 4°C.

PCR products were then isolated using a QIAGEN DNA isolation kit (QIAGEN Inc., Chatsworth, CA) and eluted with 20 μl of 10 mM Tris, 0.1 mM EDTA, pH 8.0.

DNA sequencing was performed by the dideoxychain termination method of Sanger et al. (24) using the pBluescript II SK (+) phagemid (Stratagene, San Diego, CA). For subcloning of PCR derived DNA fragments into the pBluescript vector, the phagemid was first digested with EcoRV (Boerhinger Mannheim Biochemicals, Indianapolis, IN). Addition of T's to the blunt ends of the vector was then carried out using Taq DNA Polymerase and dTTP (25, 26). PCR fragments were then annealed to the phagemid utilizing the property that PCR products synthesized by Taq DNA polymerase result in the addition of single non-templated A's to the 3' ends of the duplex DNA strands. Sequencing was carried out using the Taquence kit Version 2.0 (US Biochemical Corp., Cleveland, OH) and ^{35}S-dATP (Dupont, NEN; 10 mCi/ml) according to the manufacturers instructions.

RNA Isolation and Northern Blot Analysis

For the extraction of mRNA, the bacterial cells were harvested by centrifugation in a GS3 rotor (11,000 g, 15 min). The pelleted cells were resuspended in 20 mM sodium acetate (pH 5.5), 1 mM EDTA, 10 mM β-mercaptoethanol, 10 mM sodium dodecyl sulfate (SDS). The suspension was immediately placed in a 65°C water bath and an equal volume of hot phenol (65°C) that had been equilibrated with 20 mM sodium acetate (pH 5.5) was added. After 5 min, the suspension was subjected to centrifugation (30,000 g, 20 min) in an SS-34

rotor at 4°C to remove DNA and protein debris. The supernatant was extracted three times with phenol-chloroform (1:1), once with chloroform and precipitated by addition of 2.5 volumes of ethanol and 0.1 volume of 3M sodium acetate (pH 5.5). The RNA was washed with 80% ethanol, dried and resuspended in water.

Total RNA (20 μg) was subjected to gel electrophoresis in 1% agarose-formamide denaturing gels in 20 mM MOPS, 5 mM sodium acetate (pH 7.0), 10 mM EDTA, and transferred to nitrocellulose membranes (Schleicher and Schuell, Keene, NH) by capillary transfer (27). The filters were blocked in hybridization buffer [50% formamide, 5X SSPE (1X SSPE is 150 mM NaCl, 10 mM sodium phosphate, 1 mM EDTA), 0.1% SDS, 100 μg/ml denatured salmon sperm DNA, 5X Denhardt's solution] for 1 h. The composition of 5X Denhardt's solution was 0.1% bovine serum albumin, 0.1% polyvinlypyrolidone, and 0.1% Ficoll in H_2O. DNA probes were labeled with [α-^{32}P]dCTP (Dupont NEN, 10 mCi/ml) using random oligodeoxynucleotide primer labeling (28), and were used at 10^6 cpm/ml. The filters were incubated for 24 h at 42°C. The blots were washed three times with 2X SSPE, 0.1% SDS (15 min each), and once in 0.1X SSPE, 0.1% SDS (1 h). The washed filters were exposed to Kodak X-Omat AR film with an intensifying screen at -80°C. Relative intensities of the bands were determined by scanning densitometric analysis with a Visage 110 densitometer (Bioimage Prod., Ann Arbor, MI).

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Southern Blot Analyses

Genomic DNA was isolated by the standard phenol-chloroform method (29). The DNA (3 μg) was then digested with 20 units of EcoRI and BamHI (Boerhinger Mannheim Biochemicals, Indianapolis, IN) using reaction conditions suggested by the manufacturer. The digested DNA was electrophoresed on 0.8% agarose gels, and transferred to nytran membranes (Schleicher and Schuell, Keene, NH). The filters were then blocked in hybridization buffer and hybridized with ^{32}P-labeled probes as described for the Northern blot analyses. The filters were then washed twice in 2X SSC (1X SSC = 0.15 M NaCl and 0.5 M sodium citrate) and 0.1% SDS at 42°C for 15 min each, and once in 0.1X SSC and 0.1% SDS for 15 min at 42°C before being exposed to Kodak X-Omat AR film.

***nodD*$_1$, *glnA*, galectin-3 Oligonucleotide Probes**

The *nodD*$_1$ probe was obtained by PCR amplification of the *B. japonicum* genomic DNA using the sequences -(dATCTAAATCTTCTCGTTGCGCTC)- and -(dCGAGCAATATCCGACGCATCCAGA)- complementary to the 5' and 3' ends of the published *nodD*$_1$ sequence (18) respectively. A PCR product of 870 bp was obtained whose size was as expected from the sequence. In addition, a 270 bp stretch of the PCR product was sequenced and this segment demonstrated 100% identitity to the reported *nodD*$_1$ sequence (18). The cDNA of galectin-3 from mouse 3T3 cells was as described in Jia et al. (30).

The glutamine synthetase probe (*glnA*) was kindly provided by Dr. Greg

Martin of Purdue University, in the form of the plasmid pBJ53 (31). The plasmid was digested with EcoRI and BglII to liberate a 0.8 kb fragment from within the coding sequence, as expected from restriction map analyses. This 0.8 kb *glnA* fragment was chosen as the glutamine synthetase probe in Northern blot analyses.

RESULTS

A Molecular Probe for the BJ38 gene(s)

Coomassie blue staining of purified BJ38, that had been digested with V-8 protease and subjected to SDS-PAGE, identified four peptides: V1 (38 kd), V2 (31 kd), V3 (21 kd) and V4 (14 kd). Amino acid sequence analyses of V2 and V4 yielded partial amino acid sequences as indicated in Fig. 1A. No sequence was obtained from V1 and V3. Two degenerate primers (a sense oligonucleotide primer derived from V2 and an anti-sense primer derived from V4) were synthesized on the basis of their partial amino acid sequences and codon usage. Using genomic DNA isolated from *B. japonicum* as a template for PCR amplification, a DNA fragment (designated PCR1) of about 0.45 kb was obtained. The PCR fragment was sequenced and the deduced amino acid sequence of the PCR fragment matched exactly the experimentally determined amino acid sequence, both at the ends corresponding to the PCR primers, as well as internal regions not specified by the primers (Fig. 1B). PCR1 was thus used as an authentic probe for the BJ38 gene.

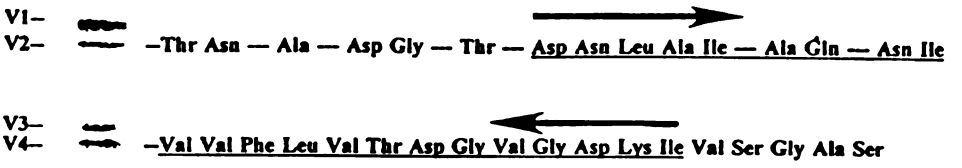
Southern and Northern Blot Analysis using the BJ38 probe

In these experiments, chromosomal DNA was first digested with the restriction endonucleases EcoRI and BamHI. These enzymes were chosen because they do not possess cleavage sites within the PCR1 probe. Southern blot analysis of the digested genomic DNA samples probed with PCR1 revealed, in each case, two

Figure 1. A) The partial amino acid sequences of peptides derived from BJ38. BJ38 was digested with V8 protease, subjected to SDS-PAGE, transferred to a Problot membrane and Coomassie blue stained. Peptides were subjected to amino acid sequence analysis and the sequences for V2 and V4 are shown at the right. The arrows highlight the amino acid sequences used to synthesize the 5'- and 3'- primers (direction indicated by the arrow) used for PCR amplification.

B) The deduced amino acid sequences of PCR1 fragment derived from the nucleotide sequence. The underlined amino acid residues represent direct matches between the amino acid sequence deduced from the nucleotide sequence of the PCR product and the experimentally determined amino acid sequence from the protein.

A. Experimentally determined peptide sequence:



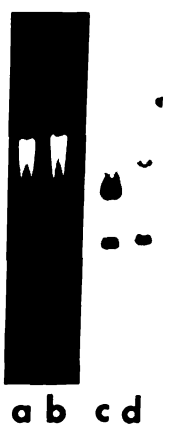
B. Deduced amino acid sequences from DNA sequence analyses:

Asp Asn Leu Ala Ile Arg Ala Gln Arg Asn Ile Thr Leu Arg Ile Asp.....

.....(140 amino acids in an open reading frame of the nucleotide sequence).....

..... Thr Pro Gln Glu Val Val Phe Leu Val Thr Asp Gly Val Gly Asp Lys Ile

Figure 2. Genomic Southern blot of *B. japonicum* DNA that had been digested with endonucleases that do not contain cleavage sites within the PCR1 probe. 3 μg of *B. japonicum* DNA was digested with 20 units of EcoRI, and BamHI and the digested DNA subjected to 0.8% agarose gel electrophoresis. DNA was transferred to Nytran membranes and the filters probed with ^{32}P-labeled PCR1. Lanes a and c , EcoRI digestion; lanes b and d, BamHI digestion. Lanes a and b, ethidium bromide staining; lanes c and d, hybridization with ^{32}P-labeled PCR1.

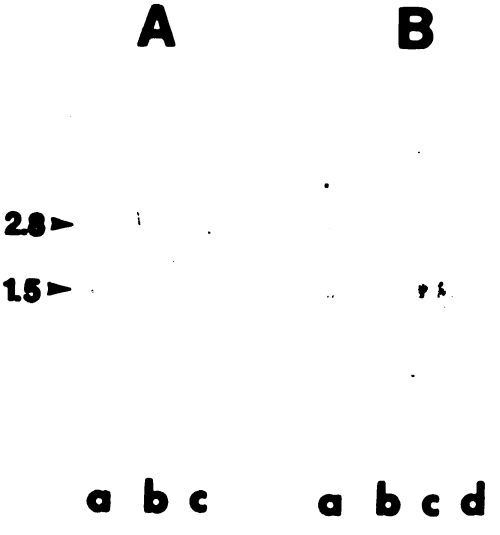

a b c d

bands (Fig. 2). These results suggest the possibility that there are either two genes for BJ38, or the presence of another gene, besides the BJ38 gene, that can hybridize with the PCR1 probe.

When Northern blots of *B. japonicum* RNA were probed with PCR1, the BJ38 probe hybridized to 2 bands of 1.5 kb and 2.8 kb (Fig. 3A, lane a). As a control, PCR1 was also used to probe RNA isolated from *R. meliloti* and *R. trifolii,* two rhizobial strains that do not bind to soybean roots. In both of these strains, no hybridization of PCR1 was observed (Fig. 3A, lanes b, c). When the *nodD*$_1$ probe was used to hybridize *B. japonicum* RNA, a single mRNA species of 1.6 kb was observed (Fig. 3B, lane b). The glutamine synthetase probe revealed a single band of ~ 1.5 kb as expected for the *glnA* transcript (Fig. 3B, lane c) (31, 32). As a negative control, the cDNA of galectin-3 from mouse 3T3 cells was used to probe the RNA blots (30). As expected, this probe showed no hybridization to *B. japonicum* RNA (Fig 3B, lane d).

The fact that the PCR1 probe hybridizes to two bands in both Southern and Northern blots suggests that there may indeed be two genes, each with its own distinct transcript. As of this writing, the sequence of the BJ38 gene (either one of the possible two) has not been completely determined. Nor is it clear whether the two mRNA species observed on the Northern blots are derived from one or distinct genes. In light of this problem, we have decided to describe the effects of saccharide and flavones on BJ38 expression in terms of both the two mRNA transcripts, hereafter, the 1.5 kb and 2.8 kb species will be designated as transcript I and transcript II, respectively.

Figure 3. A) Northern blots of RNA that had been hybridized with ^{32}P-labeled PCR1. RNA was isolated as described in the methods and materials, subjected to 1% agarose gel electrophoresis and transfered to nitrocellulose membranes. The blots were then hybridized with the ^{32}P-labeled PCR1, washed, and analyzed by audioradiography. Lanes: a) *B. japonicum*; b) *R. meliloti*; and c) *R. trifolii* RNA.
B) Northern blots of *B. japonicum* RNA that had been hybridized with: a) PCR1; b) *nodD$_1$*; c) *glnA*; d) galectin-3 probes.


A
B
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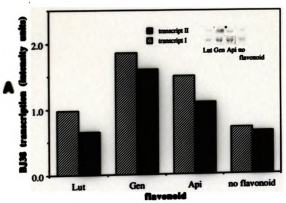
1.5
a b c
a b c d

Effect of flavonoids on BJ38 and *nodD*$_1$ expression

Our previous observation that BJ38 expression, assayed at the polypeptide level, was induced by the flavonoid genistein, a known *nod* gene inducer in *B. japonicum,* raised the possibility that BJ38 may itself be a *nod* gene. Thus the effects of flavonoids on the accumulation of mRNA for BJ38 and *nodD*$_1$ were tested. In these experiments, RNA was isolated from liquid cultures of *B. japonicum* that had been exposed to 2 μM concentrations of flavones for 10 h. This concentration of flavonoids was the same as what we had reported for genistein induction of BJ38 (9). Similarly, a 10 h exposure to flavonoids had previously been shown to be sufficient for *nod* gene induction (12). Finally, the transcript for the constitutively expressed glutamine synthetase *(glnA)* gene (33, 34) was used as an internal control in Northern blot analyses, as we had previously shown that the level of glutamine synthetase enzymatic activity was unaffected by treatment with flavonoids (9). Consistent with this previous report, the *glnA* transcript levels were not altered by the addition of flavonoids.

When RNA from *B. japonicum* was probed with PCR1, the BJ38 probe hybridized to two bands of 1.5 (transcript I) and 2.8 kb (transcript II) in both the untreated and flavonoid treated samples. The order of flavonoid induction for both transcript I and II was similar to that reported at the protein level, with genistein yielding the greatest effect (Fig. 4A). Luteolin yielded little or no effect, while apigenin yielded intermediate effects on BJ38 expression. In parallel, an examination of *nodD*$_1$ expression revealed that the *nodD*$_1$ gene was similarly induced by

Figure 4. The effect of flavonoids on the mRNA levels of BJ38 and *nodD*$_1$. Final liquid cultures were incubated for 10 h in the presence of 2 μM concentrations of genistein, apigenin, luteolin or without any flavonoid treatment. The amount of each transcript is represented in intensity units. A) BJ38 transcripts; B) *nodD*$_1$ transcript.


A
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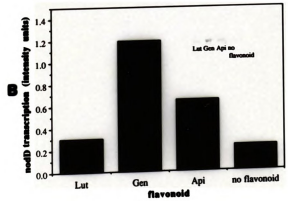
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Lut Gen Api no flavonoid
nodD transcription (intensity units)
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1.2
1.0
0.8
0.6
0.4
0.2
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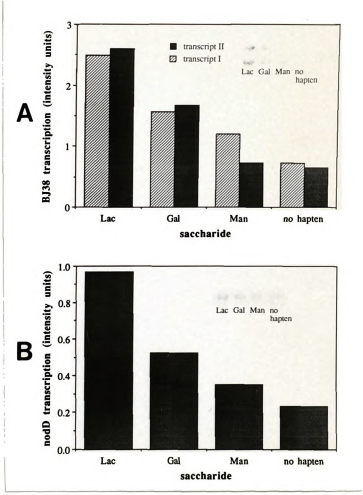
flavonoid

Figure 5. The effect of saccharides on the mRNA levels of BJ38 and *nodD*$_1$. Final liquid cultures of *B. japonicum* were incubated for 10 h in the presence of 1 mM concentrations of Lac, Gal, Man and without any saccharide. A) BJ38 transcripts; B) *nodD*$_1$ transcript.


A
BJ38 transcription (intensity units)
transcript II
transcript I
Lac Gal Man no hapten
3
2
1
0
Lac
Gal
Man
no hapten
saccharide
B
nodD transcription (intensity units)
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0.8
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flavonoids. Genistein yielded the greatest effect, followed by apigenin (Fig. 4B). Luteolin failed to have an effect on *nodD*$_1$ mRNA levels. These latter results are consistent with previously reported effects on *nodD*$_1$ expression (12, 15).

Effect of saccharides on the expression of BJ38 and *nodD*$_1$

In our previous study, we have also shown that when *B. japonicum* cells were cultured in the presence of saccharides, the amount of BJ38 isolated from these cells was elevated in an order similar to the relative affinities of the saccharides for BJ38 (9). Using the same protocol for flavone induction, the effects of saccharides on the accumulation of mRNA for BJ38 and *nodD*$_1$ were tested. Saccharides were added to a concentration of 1 mM and the cells cultured for 10 h. This 10 h length of exposure to Lac was selected as it had previously been shown to result in a 4-5- fold increase in the amount of BJ38 isolated from *B. japonicum* (9). Consistent with our previous report, the *glnA* mRNA levels were unaffected by treatment of *B. japonicum* with saccharide.

Northern blot analyses of saccharide treated samples also revealed mRNA transcripts of 1.5 kb and 2.8 kb similar to that obtained with the flavone induction (Fig. 5). The intensities of both of these bands were strongest in the Lac-treated cells, followed by Gal and mannose (Man). This relative order of induction of both transcripts was similar to that reported for saccharide induction of BJ38 at the protein level (9).

When the *nodD*$_1$ probe was used to probe the RNA samples isolated from both

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untreated and saccharide treated *B. japonicum* cells, a single *nodD*$_1$ species of ~ 1.6 kb was observed. Interestingly *nodD*$_1$ transcript levels were also found to be elevated in a saccharide dependent fashion. The order of saccharide induction was similar to that observed with BJ38 expression, with lactose yielding the greatest effect. Mannose, which had no little or no effect on BJ38 expression, had only a minimal effect on *nodD*$_1$ transcription.

DISCUSSION

In previous studies (9), we have shown that BJ38 expression at the polypeptide level could be induced by treatment of *B. japonicum* cells with either the genistein and Lac. We now document the results of a study to characterize the effect of both flavonoid compounds and saccharides on BJ38 and $nodD_1$ expression at the molecular level. The key findings of this study are (a) The levels of BJ38 mRNA transcripts were elevated by both saccharides and flavonoids in an order of efficacy similar to that reported at the polypeptide level (9); (b) $nodD_1$ induction by flavonoids demonstrated a strong correlation to that observed in the flavonoid regulation of BJ38; (c) $nodD_1$ mRNA levels were also elevated in a saccharide dependent fashion. This order of induction was: Lac > Gal > Man.

A key factor in the interpretation of these results is the validity of the BJ38 probe PCR1. This probe was obtained by PCR amplification of *B. japonicum* genomic DNA using degenerate primers derived from the partial amino acid sequences of BJ38 that had been digested with V-8 protease. PCR1 yielded a deduced amino acid sequence that matched the experimentally derived BJ38 amino sequence at both ends corresponding to the PCR primers, as well as in internal regions bounded by the primers. PCR1 was thus used as an authentic probe for BJ38. In genomic Southern blot analyses of *B. japonicum* DNA that had been digested with restriction enzymes that do not possess cleavage sites within the PCR1 probe, two

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bands were revealed. Consistent with this, two transcripts of 1.5 kb (transcript I) and 2.8 kb (transcript II) were detected in Northern blot analyses of *B. japonicum* RNA. At the present time, we do not know whether these results reflect two BJ38 genes or the presence of another gene, besides BJ38, that can hybridize to PCR1. In addition, it is also not known whether both transcript I and transcript II are derived from one or two different genes. The present results nevertheless demonstrate inducibility for both transcripts and provoke several intriguing ideas. Firstly, the observation that BJ38 can be induced by flavonoid compounds, such as genistein, raises the possibility that BJ38 is a member of the *nod* gene family. This possibility was tested by monitoring, in parallel, the levels of the $nodD_1$ transcript. $nodD_1$ was chosen as a marker for *nod* gene expression as it is inducible by flavonoids (15), as well as codes for the transcriptional regulator of other *nod* genes (18). Flavonoid induction of $nodD_1$ demonstrated a strong correlation to that observed for flavonoid regulation of BJ38. The possibility exists, therefore, that the addition of genistein to *B. japonicum* cultures results in an increased expression of the $nodD_1$ gene product, which, in turn, activates the transcription of the BJ38 gene.

Second, the observation that $nodD_1$ mRNA levels were induced by saccharides indicate alternative inducers of this gene, besides the flavonoid compounds. Consistent with this, Banfalvi et al. (15) have reported that the induction of *nod* genes with soybean exudate result in induction levels of *nod* genes greater than that obtained with the flavonoid inducers alone. As saccharides are a major component of the soybean root exudate, the possibility exists that saccharides may provide an additional

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component leading to the maximal induction of *nodD*$_1$ expression observed with soybean root exudate. Recently, Smit et al. (35) have also reported that glycoside derivatives of flavonoid compounds are actively involved in the specific induction of the *nodD*$_1$ gene. The possibility of a dual control of the *nodD*$_1$ gene by both flavonoids and saccharides in *B. japonicum* is especially intriguing in light of studies on the regulation of the *vir* genes in *Agrobacterium tumfaciens* (36, 37). In this connection, there is a striking analogy between the control of the *nod* genes of *B. japonicum* and the *vir* genes in *A. tumefaciens* (Table I). For instance, the *A. tumefaciens vir* genes necessary for the infection of the host plant are also activated by both saccharides and phenolics. This activation involves a two-component sensory transduction pathway composed of a sensor-kinase protein (VirA) and a DNA binding protein (VirG). Saccharide induction of the *vir* genes through this transduction system is mediated by the carbohydrate binding protein ChvE. When bound by its ligands, ChvE activates the sensory transduction pathway resulting in the transcription of the *vir* genes. Our present observation that the saccharide specificity of *nodD*$_1$ induction is similar to the saccharide binding specificity of BJ38 suggests the likelihood that BJ38 may serve as an analogous saccharide receptor in *B. japonicum* leading to the activation of the *nodD*$_1$ gene. If indeed *nodD*$_1$ regulation did occur via such a sensory transduction pathway, a possible candidate for both the sensor kinase and DNA binding protein components in *B. japonicum* would be the NodV and NodW proteins respectively. These proteins are essential for the full expression of the common *nodD*$_1$ and *nodYABCSUIJ* operons and have been proposed to form an

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Table I. Comparison of *A. tumefaciens vir* gene induction and *B. japonicum nod* gene induction.

	***vir* gene induction**	***nod* gene induction**
Inducer	a. saccharides: galacturonic acid > glucuronic acid > xylose > glucose b. phenolics: eg. acetosyringone	a. saccharides: Lac > Gal > Man b. flavonoids: eg. genistein
carbohydrate-binding receptor	ChvE	BJ38 ?
sensory protein	Vir A	NodV
DNA binding protein	VirG	NodW NodD

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alternative pathway for *nod* gene induction by flavonoids (38). In addition, sequence analyses of NodV have also shown it to be homologous to the VirA protein (39).

In conclusion, our present results have demonstrated that mRNA levels of BJ38 are increased by both genistein and Lac. In this connection, there is a direct correlation between the efficacy of induction by different flavonoids on BJ38 and $nodD_1$ expression. This result suggests the possibility that BJ38 may belong to the family of *nod* genes required for nodulation. As the $nodD_1$ gene product is essential for induction of *nod* genes, the effects of these compounds on BJ38 expression are also currently being tested in $nodD_1$ mutants. Studies are also in progress to sequence the BJ38 gene, and to determine if the conserved nod box sequence preceeding the inducible *nod* genes in *Rhizobium* are found in the promoter sequences of these genes. This sequence analysis will also determine whether both bands detected in Southern blots are indeed BJ38 genes, and whether both transcripts I and II are transcripts derived from one or two distinct genes.

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CHAPTER V

CONCLUDING STATEMENT

The attachment of rhizobium to the roots of the host legume is a key step in the infection process leading to the establishment of a nitrogen fixing symbiosis. When I began my thesis research, it had already been documented that, in the *Bradyrhizobium japonicum*-soybean interaction, this attachment is galactose (Gal)/lactose (Lac)-inhibitable. In addition, a carbohydrate binding protein, designated, BJ38, had been isolated from *B. japonicum.* BJ38 exhibited a pattern of specificity in saccharide binding similar, if not identical, to that of *B. japonicum* adhesion to soybean cells.

In this dissertation, I have demonstrated, using a polyclonal antiserum generated against BJ38, that the lectin is localized at one pole of the bacterium cell surface. More importantly, BJ38 localization is coincident with the site of *B. japonicum* attachment to soybean cells. Thus, the cell surface display of BJ38 is consistent with its role in mediating the polar adhesion of the bacterium to the soybean roots.

In the course of studies to obtain large amounts of BJ38 for antibody generation, the level of BJ38 expression, assayed at the polypeptide level, was found to be elevated when the bacterial cells were first cultured in the presence of

saccharides. This induction effect followed the order Lac > Gal > mannose ~ no hapten, which is similar to the relative affinities of these saccharides for BJ38. The effect of Lac and Gal on the expression of BJ38 was also observed at the mRNA level, as determined by Northern blot analysis.

Surprisingly, when the effects of flavonoids on BJ38 expression was tested, BJ38 expression was also found to be elevated, at both the transcriptional and polypeptide levels, by genistein, a known *nod* gene inducer in *B. japonicum*. This observation raised the possibility that BJ38 may be a member of the *nod* gene family required for successful nodulation. In this connection, we have also observed that the transcript levels of $nodD_1$, the transcriptional regulator of *nod* genes, were elevated by flavonoid compounds in a fashion similar to that of BJ38 induction. The possibility exists, therefore, that genistein activation of the $nodD_1$ gene results in increased levels of NodD, which then activates the transcription of the BJ38 gene. Further investigation is needed to determine if the nod box sequences preceeding the *nod* genes are found in the regulatory/promoter sequences of BJ38.

Finally, we have also shown that the levels of $nodD_1$ transcription could also be elevated by saccharide treatment of *B. japonicum*. This observation suggests that alternative inducers, besides flavonoids, may control the expression of the *nod* genes in *B. japonicum*.