Effect of Lectin on Nodulation by Wild-Type *Bradyrhizobium japonicum* and a Nodulation-Defective Mutant

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The nodulation characteristics of wild-type *Bradyrhizobium japonicum* USDA 110 and mutant strain HS111 were examined. Mutant strain HS111 exhibits a delayed-nodulation phenotype, a result of its inability to initiate successful nodulation promptly following inoculation of the soybean root. Previously, we showed that the defect in initiation of infection leading to subsequent nodulation which is found in HS111 can be phenotypically reversed by pretreatment with soybean root exudate or soybean seed lectin. This effect is not seen after pretreatment with root exudates and lectins obtained from other plant species. Treatment of strain HS111 with as little as 10 soybean seed lectin molecules per bacterium (3.3 × 10⁻¹² M) resulted in enhancement of nodule formation. Pretreatment of wild-type *B. japonicum* USDA 110 with soybean root exudate or seed lectin increased nodule numbers twofold on 6-week-old plants. Wild-type strain USDA 110 cells inoculated at 10⁴ cells per seedling exhibited a delay in initiation of infection leading to subsequent nodulation. Wild-type cells pretreated in soybean root exudates or seed lectin did not exhibit a delay in nodulation at this cell concentration. Mutant strain HS111 pretreated in seed lectin for 0 or 1 h, followed by washing with the hapten D-galactose to remove the lectin, exhibited a delay in initiation of nodulation. Phenotypic reversal of the delayed-nodulation phenotype exhibited by strain HS111 was seen if incubation was continued for an additional 71 h in plant nutrient solution following 1 h of lectin pretreatment. Reversal of the delayed-nodulation phenotype of HS111 through lectin pretreatment was prevented by chloramphenicol or rifampin. These studies indicate that the presence of soybean lectin can affect the nodulating ability of wild-type *B. japonicum* as well as that of mutant HS111. Apparently, the lectin induces de novo protein synthesis in *B. japonicum*, which is required for efficient initiation of the nodulation process. Our working hypothesis is that, in the presence of the plant host (lectin), the proportion of the population of *B. japonicum* cells capable of efficient nodulation is increased.

The molecular mechanisms of recognition between rhizobia and legumes involved in the establishment of an effective nitrogen-fixing symbiosis can be considered a form of cell-cell communication. These cellular interactions initiate a complex developmental process whereby the rhizobia adhere to and penetrate the root, inducing differentiation of cortical root cells into a nodule. Rhizobia reside in the nodule and reduce atmospheric nitrogen to ammonia, which can be utilized by the plant. A prevalent hypothesis to explain the mechanism of recognition between symbiont and host has been the specific binding of compatible rhizobia to host plant root lectins (1, 10, 13, 17, 26-28). Lectins are noncatalytic proteins which are capable of specific interaction with microbial cell surface carbohydrates (1, 27).

In previous work, the mechanism of recognition in the *Bradyrhizobium japonicum*-soybean symbiosis was investigated by using a mutant of *B. japonicum*, strain HS111, which exhibits a delayed-nodulation phenotype (16, 17, 29). The nodulation phenotype of mutant strain HS111 is the result of its inability to promptly initiate infection leading to subsequent nodulation. The phenotype was determined by a bioassay (9) in which the rate of nodule initiation is measured relative to the position of the primary root tip (RT) at the time of inoculation (16, 17). The RT and smallest emerging root hair are marked on the surface of plastic growth pouches with the aid of a 10× binocular microscope (9, 16, 17). This area, marked at the time of inoculation, is the region most susceptible to infection (23) and nodulation (9, 16). Owing to transient acropetal development of the root, this area remains susceptible to nodulation for only 4 to 6 h (6, 9). Therefore, the relative rate at which successful infections are initiated can be determined by the location of nodules in relation to the RT mark made at the time of inoculation. That is, if nodules only form significantly below the RT mark, then successful initiation of nodulation is delayed. The average distance of the uppermost nodule formed by wild-type strain USDA 110 is approximately 2 mm above the RT mark (16, 17). Strain USDA 110 forms ≥67% of its nodules above the RT mark (16, 17). In contrast, mutant strain HS111 generally forms nodules (≥60%) well below the RT mark (16, 17). The average distance of the uppermost nodule formed by strain HS111 is approximately 18 mm below the RT mark (16, 17). Therefore, mutant strain HS111 is defective in its ability to initiate successful infections (i.e., leading to nodule formation) in the RT-smallest emerging root hair zone.

Delayed initiation of nodulation by mutant strain HS111 suggests that nodulation can occur only after cells have been stimulated in the rhizosphere (16). The defect in initiation of nodulation in HS111 can be phenotypically reversed by simulating the plant rhizosphere through preincubation with soybean root exudates (16), soybean seed lectin (SBL), or a galactose-binding, root-excreted protein(s) prior to inoculation (17). A soybean root lectin which exhibits an amino acid composition and hapten specificity for D-galactose similar to those of the seed lectin has been isolated (14). mRNA

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isolated from soybean roots, when translated in vitro, produces a protein immunologically cross-reactive with SBL (25). We suggested previously (17) that the galactose-binding root protein(s) which reverses the delayed nodulation phenotype of HS111 could be a galactose-specific root lectin (14, 25). The presence of a galactose-binding protein(s) in root exudates and its effect on the nodulation phenotype of strain HS111 suggests that it may act prior to root attachment, perhaps by inducing competence for nodulation in the respective Bradyrhizobium sp. (17). Although our previous work indicated a clear effect of lectin on the nodulating ability of mutant HS111, several questions remained. For example, is the effect of lectin only exhibited on mutant cells, not on the wild type? What is the specificity of the response of the mutant or wild type to lectin? What is the nature of the physiological response of B. japonicum to lectin? This report addresses these questions.

MATERIALS AND METHODS

Bradyrhizobium cultures and preparation of inocula. Wild-type B. japonicum USDA 110 was originally obtained from G. Elkan (North Carolina State University, Raleigh) and is colony type II10 (19). Isolation of slow-to-nodulate mutant strain HS111 and characterization of its nodulation phenotype have been reported previously (16, 17, 29).

Bradyrhizobium cultures were maintained on YEM agar (19) and grown to mid-log phase (3.5 days) at 30°C in YEM broth as described previously (16, 17). Cell cultures were pelleted by centrifugation at 7,000 × g for 10 min, washed with 10 ml of sterile, sucrose-free, half-strength plant nutrient solution (PNS; 30), and suspended to a cell density of either 10⁶ or 2 × 10⁶ cells per ml (4, 9, 16, 17) in half-strength PNS. Sucrose-free, half-strength PNS was used in all experiments. Samples of these suspensions were used to inoculate plants directly (10⁵ cells per ml) or for pretreatment inoculations (2 × 10⁶ cells per ml).

Plant and lectin varieties. The soybean (Glycine max (L.) Merr.) used was the cultivar Essex obtained from D. R. Mayo Seed Co., Knoxville, Tenn. Three different cowpea (Vigna unguiculata (L.) Walp.) cultivars were utilized: Knuckle Crowder Hull, California Blackeye Pea, and Mississippi Silver Hull. The alfalfa (Medicago sativa (L.)) cultivar Buffalo, pea (Pisum sativum (L.)) cultivar Wanda Sweet Pea, and the tomato (Lycopersicon esculentum) were all obtained from Mayo. The SBL and jackbean lectin (concanavalin A) were obtained from E. Y. Laboratories, San Mateo, Calif. The N-acetylglucosamine- or D-galactose-specific lectins from peanut (Arachis hypogaea), horse gram (Dolichos biflorus), and castor bean (Ricinus communis) were obtained from Sigma Chemical Co., St. Louis, Mo.

Growth and inoculation of seedlings. Soybean seeds were surface sterilized and germinated as described previously (16, 31). Seedlings (2 days old) were transferred aseptically to autoclaved clear plastic growth pouches (diSPo Seed Pack; Northrup King Seed Co., Minneapolis, Minn.), which had been moistened with sucrose-free, half-strength PNS prior to autoclaving (16, 17). Seedlings were maintained in a growth chamber under the following conditions: 26°C; light intensity, 510 microeinsteins m⁻² s⁻¹; a 15-h photoperiod. Pouches were watered with sterile water as needed. The positions of the RT and smallest emerging root hair of 3-day-old seedlings were marked on the surface of the pouch with the aid of a dissecting microscope (9, 16, 17). The entire root of each seedling was inoculated dropwise with 1.0 ml of a 10⁸ cell per ml suspension. Each pouch contained three seedlings, and uninoculated pouches were included with each experiment to monitor for contamination.

Growth of plants for collection of root exudates. Aseptically germinated 2-day-old soybean, cowpea, alfalfa, and pea seedlings were fixed atop a fluted paper wick in a sterile 45-ml serum vial containing 15 ml of sucrose-free, half-strength PNS (16, 17). The seed coats of 2-day-old cowpea seedlings were aseptically removed to allow for growth of a straight radicle. For tomato, radicles did not appear until day 5, and seedlings then were placed in the serum vials. A sterile, 18-oz (1 oz = 29.573 ml) Whirlpac bag (Nasco Inc., Oakville, Conn.) was placed over each vial. The vials were then placed in a growth chamber for 10 days (16, 17). The half-strength PNS containing the root exudates was pooled and prepared for Bradyrhizobium pretreatments as described previously (5, 16, 17).

Pretreatment of B. japonicum with root exudate and lectin. B. japonicum cultures were prepared as described above. Ten milliliters of the 2 × 10⁶ cell per ml Bradyrhizobium suspensions were aseptically added to 50 ml of root exudate; sucrose-free, half-strength PNS; or sucrose-free, half-strength PNS containing 10 μg of lectin per ml in sterile 250-ml Erlenmeyer flasks. Following preincubation at 30°C without shaking for 36 h, cells were harvested by centrifugation, washed, and suspended to a concentration of 10⁸ cells per ml. For some experiments, log dilutions of the original cell suspension were done in sterile, half-strength PNS before inoculation onto plants. Plate counts were performed to determine the number of viable cells.

Effect of lectin concentration on nodulation enhancement. A 10-μg/ml (1.2 × 10⁶ molecules of SBL per bacterium) solution was prepared in half-strength PNS. This solution was serially diluted into 50-ml samples of half-strength PNS to give a lectin level from the original (1.2 × 10⁵ molecules of SBL per bacterium) down to 10⁻⁵ molecules of SBL per bacterium. Ten milliliters of a 2 × 10⁹ cell per ml strain HS111 cell suspension was aseptically added to each 50-ml sample. These cell suspensions were prepared for inoculation as described above.

Hapten inhibition of nodulation enhancement. Cells of strain HS111 pretreated for 1 + 71 h were incubated for 1 h in the presence of SBL and then washed twice with sterile, sucrose-free, half-strength PNS containing 30 mM D-galactose. The cells were suspended in sterile, half-strength PNS and incubated for an additional 71 h (i.e., preincubation for 1 + 71 = 72 h total). Strain HS111 pretreated for only 1 h in the presence of SBL was harvested by centrifugation, washed twice with sterile, half-strength PNS containing 30 mM D-galactose, and suspended in half-strength PNS prior to inoculation of seedlings. Pretreatment for 0 h involved suspending the cells in the presence of SBL, centrifuging immediately, and then washing and suspending the cells as in the other treatments.

Chloramphenicol and rifampin pretreatment. Mutant strain HS111 was grown to mid-log phase from single-colony isolates in 50 ml of modified Bergersen minimal medium (3; MM) containing 5.4 × 10⁻² M glycerol and 5.9 × 10⁻³ M sodium glutamate. These cultures were grown in a rotary shaker (Queue Systems, Parkersburg, W. Va.) at 30°C for 4 days. Cell cultures were prepared for pretreatment as described above except that they were suspended in sterile MM with or without 10 μg of SBL per ml. Pretreatment solutions contained either 25 μg of rifampin or 50 μg of chloramphenicol per ml. These concentrations had been previously found to inhibit growth of B. japonicum. Solu-
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TABLE 1. Nodulation characteristics of mutant strain HS111 pretreated with root exudates obtained from various plant species

<table>
<thead>
<tr>
<th>Pretreatment or root exudate source</th>
<th>Cultivar</th>
<th>Avg distance (mm) of uppermost nodule from RT ± SEM</th>
<th>% Nodulation only below RTa</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vigna unguiculata</td>
<td>Knuckle Crowder Hull</td>
<td>-18.0 ± 3.8</td>
<td>76</td>
</tr>
<tr>
<td>Vigna unguiculata</td>
<td>California Blackeye Pea</td>
<td>-18.7 ± 4.5</td>
<td>80</td>
</tr>
<tr>
<td>Vigna unguiculata</td>
<td>Mississippi Silver Hull</td>
<td>-20.3 ± 2.5</td>
<td>81</td>
</tr>
<tr>
<td>Medicago sativa</td>
<td>Buffalo</td>
<td>-14.2 ± 3.5</td>
<td>62</td>
</tr>
<tr>
<td>Pisum sativum</td>
<td>Wanda Sweet Pea</td>
<td>-17.4 ± 3.5</td>
<td>77</td>
</tr>
<tr>
<td>Lycopersicon capsulatum</td>
<td>Essex</td>
<td>-20.5 ± 3.5</td>
<td>77</td>
</tr>
<tr>
<td>Glycine max</td>
<td>Essex</td>
<td>-1.1 ± 1.8</td>
<td>38</td>
</tr>
<tr>
<td>PNS</td>
<td></td>
<td>-20.0 ± 4.1</td>
<td>76</td>
</tr>
<tr>
<td>SBL (10 µg/ml)</td>
<td></td>
<td>-1.5 ± 2.4</td>
<td>32</td>
</tr>
</tbody>
</table>

a Preincubation was for 72 h. Values are averages from 31 to 46 plants per experiment. Each experiment was repeated one or more times.

b Minus signs indicate nodulation below the RT mark made at the time of inoculation.

tions were incubated at 30°C for 72 h prior to preparation for inoculation of seedlings as described above. The number of viable cells was determined before and after the 72-h incubation period. Preincubation of strain HS111 in MM for 72 h without antibiotics resulted in a substantial increase in cell number. Pretreatment with either chloramphenicol or rifampin reduced the number of viable cells by approximately 10% compared with the original inoculum. Suspensions were prepared for inoculation as described previously and suspended to the standard inoculum concentration of 10⁸ cells per ml.

Nodule scoring of pouch assay. Plants inoculated with wild-type B. japonicum USDA 110 were scored for nodulation 14 days after inoculation and the slow-to-nodulate mutant strain HS111 was scored 28 days after inoculation. Scoring wild-type nodulation at 28 days did not affect the results. The positions of all nodules on the primary root were measured to the nearest 0.1 mm relative to the RT mark made on the surface of the plastic growth pouch at the time of inoculation. All experiments were repeated at least twice unless mentioned otherwise in the text.

Enhanced nodulation by lectin pretreatment. Three-day-old, surface-sterilized soybean seedlings, as described above, were aseptically removed from plastic growth pouches and placed in 6-in. (1 in. = 2.54 cm) plastic azalea pots filled with vermiculite moistened with half-strength PNS. Wild-type B. japonicum USDA 110 was pretreated with either sterile, half-strength PNS containing 10 µg of SBL per ml; soybean root exudate; or sterile, half-strength PNS. Three seedlings were placed in each pot, and each seedling was inoculated with 1.0 ml of a 10⁶ cell per ml suspension of wild-type cells. Plants were grown in a growth chamber under the growth conditions described above (see Table 3, experiment 2) or under the following conditions: 320 micromoleinsts m⁻² s⁻¹ with a 14-h photoperiod for 6 days/week (see Table 3, experiment 1). Plants were watered as needed with half-strength PNS for 2 weeks and then with sterile water for the duration of the experiment. After 6 weeks of growth, the plants were scored for nodulation by counting the number of nodules per plant and determining the fresh weight (in milligrams) of nodules per plant.

Adsorption and root hair curling (Hac) of rhizobia. Adsorption of B. japonicum USDA 110 and HS111 to soybean roots was determined by a modification of the procedure developed by Pueppke (24). Bacterial cultures were grown to mid-log phase, pelleted, washed, and suspended to an approximate cell concentration of 2 × 10⁸ cells per ml in half-strength PNS. Three-day-old seedlings were suspended in the inoculum for 1 h. Seedlings were washed three times in sterile, half-strength PNS by shaking on a Queue rotary shaker at 75 rpm for 15 min. The distal 20-mm segment of each root was excised, and the tissue was homogenized in 1.0 ml of PNS. The homogenized solution was serially diluted and plated to determine the number of bacteria bound per root segment. Root hair curling by B. japonicum was observed as described by Pueppke (23).

RESULTS

Lectin specificity in nodulation enhancement. Previously, we demonstrated that preincubation in soybean root exudates from a variety of soybean cultivars resulted in reversal of the slow-to-nodulate phenotype of mutant strain HS111 (17). B. japonicum has been shown to nodulate cowpea (V. unguiculata (L.) Walp.) (29). Mutant strain HS111 was preincubated in root exudates from cowpea and other legumes to determine whether these root exudates have the ability to reverse the mutant phenotype. The data in Table 1 indicate that pretreatment of mutant strain HS111 with the legume root exudates tested (cowpea, alfalfa, and pea) did not induce phenotypic reversal of the mutation of strain HS111. B. japonicum USDA 110 and HS111 nodulated the cowpea varieties used to obtain root exudates. In addition, preincubation with root exudates obtained from tomato (L. capsulatum) did not affect the nodulation characteristics of mutant strain HS111 (Table 2).

The active factor in soybean root exudates which phenotypically reverses the defect in nodulation in mutant strain HS111 has been demonstrated to be a galactose-binding protein (17). Pretreatment of mutant strain HS111 with certain N-acetylglucosamine- and D-galactose-specific lectins isolated from various plant species other than soybean did not reverse the nodulation defect of HS111 (Table 2). Kamberger (18) demonstrated by Ouchterlony double diffusion that jackbean (Canavalia ensiformis) lectin (concanavalin A) binds to the cell surface of B. japonicum. The data in Table 2 show that pretreatment of strain HS111 with concanavalin A did not phenotypically reverse its mutation. Tables 1 and 2 demonstrate that only the N-acetylgalactosamine- and D-galactose-specific lectins isolated from soybean root exudates or seeds had the ability to enhance the nodulation characteristics of mutant strain HS111.

Effect of lectin concentration on nodulation enhancement. The affinity constant for the binding of SBL to receptors on B. japonicum USDA 138 has been determined to be approximately 4 × 10⁻⁷ M (8). Binding curves of lectin to strain USDA 110 are similar to that of strain USDA 138 (8). The maximum number of SBL molecules binding to B.
TABLE 2. Nodulation characteristics of mutant strain HS111 pretreated with various lectins

<table>
<thead>
<tr>
<th>Pretreatment or lectin source*</th>
<th>Sugar specificity</th>
<th>Avg distance (mm) of uppermost nodule from RT mark ± SEM†</th>
<th>% Nodulation only below RT‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>PNS</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glycine max (SBL)</td>
<td>N-Acetylgalactosamine, α-D-galactose</td>
<td>−18.8 ± 4.1</td>
<td>71</td>
</tr>
<tr>
<td>Arachis hypogaea</td>
<td>D-Galactose</td>
<td>−19.1 ± 3.7</td>
<td>69</td>
</tr>
<tr>
<td>Dolichos biflorus</td>
<td>N-Acetylgalactosamine</td>
<td>−16.7 ± 3.5</td>
<td>77</td>
</tr>
<tr>
<td>Ricinus communis</td>
<td>α-D-Mannopyranosides, α-D-glucopyranosides</td>
<td>−18.5 ± 3.4</td>
<td>65</td>
</tr>
<tr>
<td>Canavalia ensiformis</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Each lectin was used at a concentration of 10 μg/ml.
† Values are averages from 27 to 43 plants per experiment. Each experiment was replicated one or more times.
‡ Minus signs indicate nodulation below the RT mark made at the time of inoculation.

japonicum USDA 110 ranges from $10^5$ to $10^6$ per cell (8, 30). Therefore, a lectin concentration of 10 μg/ml ($1.2 \times 10^5$ lectin molecules per cell), used in this and previous studies (16, 17), would approach saturation of the lectin binding sites of the cell. Strain HS111 was pretreated in various concentrations of lectin to determine the minimum concentration necessary to induce the physiological response. A lectin concentration of 10 molecules per cell ($3.3 \times 10^{-12}$ M) resulted in half-maximal response (Fig. 1). This value is statistically different from lectin concentrations of $10^{-2}$, $10^{-3}$, and 0 molecules per cell at a $P = 0.01$ confidence level. Statistical analysis was performed by using the Student-Newman-Keuls test based on unequal sample sizes. At a concentration of $10^{-1}$ lectin molecules per cell, a small percentage of the cell population was phenotypically affected (Fig. 1). With increasing lectin concentration, a greater percentage of the mutant population was induced to initiate nodulation at rates comparable to wild-type levels (Fig. 1).

Enhanced nodulation of wild-type B. japonicum USDA 110 by lectin. Soybean root exudate or SBL had no apparent effect on the nodulation characteristics of the wild type when measured by pouch assay (16, 17). It was, therefore, necessary to show through other assay techniques whether or not lectin has an effect on the nodulation characteristics of the wild type. Pretreatment of mutant strain HS111 with soybean root exudate increased nodule numbers approximately twofold in pouch experiments (16). The effect of lectin pretreatment on the number of nodules formed on soybean by strain USDA 110 was determined by growing the plants in pots containing vermiculite for 6 weeks. SBL or soybean root exudate pretreatment increased the total number of nodules formed by the wild type (Table 3). Soybean plants in experiment 1 (Table 3) were grown under a shorter photoperiod as described in Materials and Methods. The average fresh weight of the roots, stems, and leaves per plant was not affected by the increase in nodule number (data not shown). The average number of nodules on the primary and secondary roots increased with lectin or root exudate pretreatment (Table 3, experiment 2).

Effects of lectin on the population of wild-type cells. The degree of nodulation above the RT mark, corresponding to various inoculum concentrations, can provide clues regarding the possible threshold levels required for initiation of nodulation (7, 9). Phenotypic reversal of the delay in initiation of nodulation in HS111 by lectin pretreatment (16, 17) suggests that lectin mediates the induction of nodulation competence. To test this hypothesis, dose-response curves were determined for wild-type B. japonicum USDA 110 pretreated with lectin, root exudate, or PNS. Sets of 60 seedlings were inoculated with 1.0 ml of a pretreated cell suspension at various concentrations. The number of viable cells per seedling was determined for each serial dilution by plate counts. The dose-response curves for B. japonicum USDA 110 are shown in Fig. 2.

The average distance of the uppermost nodule from the RT mark is an indicator of the relative rate of initiation of successful nodulation (6, 7, 9, 16, 17). At a concentration below $10^6$ cells per ml, wild-type cells in PNS exhibit a delay in initiation of nodulation similar to that of mutant strain HS111 (16, 17). The average distance of the uppermost nodule migrated farther below the RT mark with lower cell concentrations (Fig. 2). Lectin or root exudate pretreatment...
TABLE 3. Effect of root exudate and SBL pretreatment on the nodulation characteristics of wild-type *B. japonicum* USDA 110

<table>
<thead>
<tr>
<th>Pretreatment</th>
<th>Expt 1*</th>
<th>Expt 2*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Sample size</td>
<td>Avg no. of nodules ± SEM</td>
</tr>
<tr>
<td>PNS</td>
<td>42</td>
<td>17.5 ± 1.3</td>
</tr>
<tr>
<td>Root exudate</td>
<td>40</td>
<td>41.2 ± 2.7</td>
</tr>
<tr>
<td>SBL*</td>
<td>38</td>
<td>39.8 ± 2.3</td>
</tr>
</tbody>
</table>

* Plants were grown under different growth conditions in the two experiments. Statistical analysis of the data by the Student-Newman-Keuls test based on unequal sample sizes demonstrated a significant difference between PNS and root exudate or SBL at a *P* = 0.01 confidence level for all experimental variables.
* SBL was used at a concentration of 10 μg/ml.

Effect of lectin on the wild type was similar to that seen with mutant strain HS111.

**Effect of preincubation time on nodulation enhancement.** Previously we demonstrated that a half-maximal nodulation response occurs with 1-h pretreatments in lectin or root exudate (17). These experiments, however, involved an initial 1 h of preincubation followed by an additional 71 h before inoculation onto plants. Initially, we interpreted these data as suggesting that the amount of time necessary for strain HS111 to interact with lectin and produce an effect on nodulation was very short. However, nodulation enhancement did not occur after 0 or 1 h of incubation in the presence of SBL without additional incubation in the absence of SBL (Table 4). A half-maximal response was

![Figure 2](http://aem.asm.org/)

**Figure 2.** Effect of inoculum concentration and lectin pretreatment on the relative rate of nodulation by *B. japonicum* USDA 110. Sets of 60 seedlings were inoculated with different cell concentrations and scored for nodulation 14 days later. Plate counts were performed to determine the actual cell concentration of the inoculum. Symbols: ○, USDA 110 PNS; ▲, USDA 110 root exudate; ■, USDA 110 SBL. Statistical analysis of the data by the Student-Newman-Keuls test based on unequal sample sizes demonstrated a statistically significant difference in the average distance of the uppermost nodule from the RT mark between PNS-root exudate- or PNS-SBL-treated and untreated (PNS) cells at a *P* = 0.01 confidence level for concentrations of 10^2, 10^3, and 10^4 cells per seedling. In the cases of PNS and SBL data, the results represent the average of two experiments.
initiated following 1 h of pretreatment if the cells were allowed to incubate for an additional 71 h after lectin removal (Table 1; 16). Therefore, a 1-h period of lectin-cell interaction is sufficient to induce a faster nodulation response, provided that time is allowed for a change in the physiology of the cells.

**Adsorption and root hair curling of* B. japonicum.** Root exudate pretreatment of strain HS111 did not affect its root adsorption ability (0.8 ± 0.35% of the cell population bound per root segment) compared with strain HS111 treated in PNS (0.64 ± 0.21%) or wild-type strain USDA 110 (0.68 ± 0.24%). Values are averages of three experiments with at least 10 seedlings per strain. These data are consistent with the observations made by Pueppke (24), who demonstrated that SBL pretreatment or root culturing of rhizobia did not affect adsorption to soybean root surfaces. The root hair curling (Hac) characteristics of wild-type strain USDA 110 and mutant strain HS111 were comparable; neither lectin nor root exudate pretreatment of strain HS111 affected root hair curling ability (data not shown).

**Requirement of de novo protein synthesis for nodulation enhancement.** The previous data suggest that enhancement of nodulation by* B. japonicum* produced by lectin pretreatment is not due simply to adsorption of lectin to the cell surface. Instead, the data suggest that lectin induces a physiological change in the cells. To determine whether lectin induces RNA or protein synthesis, we sought to test the effect of lectin on the nodulation ability of mutant strain HS111 in the presence of rifampin or chloramphenicol. Lectin pretreatment of strain HS111 in PNS in the presence of the antibiotic rifampin or chloramphenicol, however, resulted in a drastic reduction in the number of viable cells. The loss of viability of cells treated with antibiotics in PNS was likely due to the fact that they were in a nutrient-poor medium. Therefore, to prevent cell death, strain HS111 was pretreated in sterile MM (3) containing SBL and chloramphenicol or rifampin for 72 h. Under these conditions, there was little loss of viability. Following the 72-h preincubation period with or without antibiotics, the cells were suspended to a concentration of 10^8 cells per ml and inoculated onto plants. Both chloramphenicol and rifampin inhibited the nodulation-enhancing characteristics of SBL on mutant strain HS111 (Table 5). Similar results were obtained with the wild-type strain (data not shown).

**DISCUSSION**

Previously, we demonstrated that a galactose-binding protein excreted from roots, obtained from a variety of soybean cultivars, is capable of phenotypically reversing the defect in mutant strain HS111 in initiating a prompt, successful nodulation response (17). The presence of a galactose-specific soybean root lectin which is genetically distinct from SBL but shares the same hapten specificity has been documented (14, 15, 25). This may explain the previous correlations between SBL binding to* Bradyrhizobium* spp. and nodulation (5, 8, 10, 13). The response of mutant strain HS111 to SBL is specific. Neither other legume and nonlegume root exudates (Table 1) nor d-galactose- and N-acetylglucosamine-specific lectins (Table 2) are capable of phenotypically reversing the mutation. Subsaturation concentrations of lectin (10^1 molecules of lectin per cell [Fig. 1]) are capable of initiating a faster rate of nodulation by mutant strain HS111. Lectin need only react with the* Bradyrhizobium* cell surface for a relatively short period (1 h) to induce a faster nodulation response, provided that additional time is allowed for a physiological change in the cell.

The effect of lectin on the nodulation characteristic of wild-type* B. japonicum* USDA 110 was demonstrated by two independent methods (Table 2; Fig. 2). Plants grown in vermiculite and inoculated with root exudate or lectin-pretreated wild-type cells produced a twofold greater number of nodules. Inoculation of seedlings with serial dilutions of* B. japonicum* at a concentration of 10^6 cells per seedling or lower produces a delayed nodulation pattern similar to that of mutant strain HS111 (16, 17). The data in Fig. 2 suggest that only a percentage of PNS-pretreated wild-type cells were in the proper physiological state to initiate a rapid nodulation response. Bhuvaneswari et al. (7) presented similar observations and demonstrated that culture age affects the rate of nodulation above the RT mark. Those authors suggested that culture age probably affects the efficiency of the initiation of infection. Following root exudate or lectin pretreatment, however, a greater percentage of the cell population could promptly initiate a nodulation response (Fig. 2). Bhuvaneswari et al. (7) demonstrated a direct correlation between the percentage of plants nodulating above the RT mark and the percentage of cells binding to soybean lectin with changing culture age. Here we have demonstrated that lectin addition induced competence for nodulation in* B. japonicum* which resulted in a faster rate of initiation of successful nodulation.

Either chloramphenicol or rifampin prevents reversal of the defective-nodulation phenotype of strain HS111 by lectin. This suggests that SBL functions as a signal molecule which induces de novo protein synthesis necessary for nodulation. Recently, other laboratories have presented evidence for the involvement of plant extracts or root

**TABLE 4. Effect of SBL preincubation time on nodulation enhancement of mutant strain HS111**

<table>
<thead>
<tr>
<th>Pretreatment</th>
<th>Time of incubation (h)</th>
<th>Avg distance (mm) of uppermost nodule from RT mark ± SEM</th>
<th>% Nodulation only below RT</th>
</tr>
</thead>
<tbody>
<tr>
<td>PNS</td>
<td>1 ± 71</td>
<td>-16.2 ± 2.9</td>
<td>69</td>
</tr>
<tr>
<td>SBL</td>
<td>72</td>
<td>-1.0 ± 1.2</td>
<td>33</td>
</tr>
<tr>
<td>SBL</td>
<td>0</td>
<td>-19.1 ± 3.2</td>
<td>68</td>
</tr>
<tr>
<td>SBL</td>
<td>1</td>
<td>-16.8 ± 2.7</td>
<td>74</td>
</tr>
<tr>
<td>SBL</td>
<td>1 ± 71</td>
<td>-9.3 ± 2.5</td>
<td>67</td>
</tr>
<tr>
<td>Root exudate</td>
<td>1 ± 71</td>
<td>-10.6 ± 2.4</td>
<td>64</td>
</tr>
</tbody>
</table>

* SBL was used at a concentration of 10 μg/ml.

**TABLE 5. Effect of chloramphenicol and rifampin on the nodulation-enhancing characteristics of SBL in HS111**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Avg distance (mm) of uppermost nodule from RT mark ± SEM</th>
<th>% Nodulation only below RT</th>
</tr>
</thead>
<tbody>
<tr>
<td>MM</td>
<td>-19.5 ± 4.3</td>
<td>78</td>
</tr>
<tr>
<td>SBL</td>
<td>-1.8 ± 2.7</td>
<td>39</td>
</tr>
<tr>
<td>MM-chloramphenicol</td>
<td>-24.3 ± 3.5</td>
<td>85</td>
</tr>
<tr>
<td>SBL-chloramphenicol</td>
<td>-18.9 ± 4.2</td>
<td>69</td>
</tr>
<tr>
<td>MM-rifampin</td>
<td>-19.8 ± 5.4</td>
<td>63</td>
</tr>
<tr>
<td>SBL-rifampin</td>
<td>-16.7 ± 4.8</td>
<td>64</td>
</tr>
</tbody>
</table>

* SBL was used at 10 μg/ml.

† Minus signs indicate nodulation below the RT mark made at the time of inoculation.

‡ Contained 25 μg of rifampin or 50 μg of chloramphenicol per ml.
EFFECT OF LECTIN ON NODULATION

exudates in inducing gene expression (20–22). Olson et al. (22) reported that Rhizobium fredii promoters fused to the lacZ gene showed increased β-galactosidase levels when cells were grown in the presence of soybean or kidney bean root extracts, but not with root extracts obtained from other legumes or nonlegumes. The identity of the factor(s) which induces gene expression was not reported. Recently, Mulligan and Long (20) reported that R. meliloti nodC::lacZ fusions are induced by alfalfa plant extracts. The active factor in the plant extracts is heat stable and of low molecular weight. These data correspond to our observations that soybean root exudate or SBL functions in inducing B. japonicum gene expression necessary for nodulation (Tables 1 and 2). The virC (virulence) promoter of Agrobacterium tumefaciens fused to lacZ is induced by plant exudates from several dicotyledonous plants (21). Host plant extracts also induce a change in the morphology of the smut fungus, Ustilago violacea, leading to the induction of parasitic stages in previously saprophytic cells (11, 12). The inducer is absent in most plants outside the host family but is almost universally present within species of the host family (11, 12). These examples demonstrate the growing awareness of the presence and specificity of host-produced factors involved in the induction of microbial gene expression in plant-microbial interactions.

The manner in which lectin mediates recognition in the Rhizobium-legume symbiosis has been thoroughly discussed (1, 2, 13, 26, 27). Dazzo and Hubbell (17) suggested that the clover lectin and an R. trifolii cell surface receptor function in determining host specificity and root adherence through formation of a bridge between the host and rhizobia (contact recognition). If this model is correct, one might expect that the addition of lectin would increase Bradyrhizobium adsorption to the root, perhaps resulting in a faster rate of nodulation. Lectin pretreatment of mutant strain HS111, however, did not increase adsorption to the root surface. Lectin binding to B. japonicum is saturated within 1 h (8); therefore, if lectin binding is necessary for enhancement of nodulation, a 1-h incubation period should be sufficient. Short incubation periods in the presence of lectin do not affect the nodulation properties of mutant strain HS111. It is important to remember, however, that R. trifolii and B. japonicum are quite different bacteria that infect different hosts. The mechanism used for infection could differ among rhizobium-host combinations. Apparently, lectin binding to B. japonicum induces a physiological response which enhances the rate of nodulation beyond the root hair curling stage. Mutant strain HS111, without lectin pretreatment, is Hae + at a level comparable to that of the wild-type strain. Furthermore, lectin or root exudate pretreatment of strain HS111 did not alter hair curling ability. It is possible that lectin is involved in inducing infection thread formation or in maintenance of the infection thread. The percentage of abortive infections could be an important factor in determining nodule formation above the RT mark.

Recognition in the Rhizobium-legume symbiosis is most likely not a one-step process but the cumulative effect of a series of signal and response interactions (2). Lectin induction of nodulation competence in B. japonicum is a possible example of one of these interactions. In such a model, B. japonicum would interact with the soybean lectin on the root surface or in the rhizosphere, resulting in induction of a physiological process necessary for prompt nodulation. Lectin would not necessarily be the sole determinant of binding to the root surface in this model. Other factors could be involved in the attachment process or in enhancing nodulation competence or both. How lectin functions as a signal molecule is unknown; a secondary signal following initial lectin binding to the cell surface may be necessary to mediate gene expression.

ACKNOWLEDGMENTS

We thank Robert Zabloutowicz for helpful discussions and sharing information prior to publication. We also thank Otto Slater for editorial comments on the manuscript.

This work was supported by Public Health Service grant 1-R01 GM 33494-01A1 from the National Institutes of Health and a grant from the U.S. Department of Agriculture (84-CRCR-1-1419).

LITERATURE CITED