Rapid, multiphasic attachment of *Bradyrhizobium japonicum* soybean roots

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Abstract

Bradyrhizobium japonicum strains 2143 and 110 were found to attach very rapidly and firmly to soybean roots in a multi-phasic manner. Two distinct phases of attachment were observed, plus possibly two additions phases, within the first 5 minutes of attachment. The first phase of attachment occurred within 30–45 seconds and sonication was required to remove these bacteria from the roots. The second phase was characterized by a decline in the number of bacteria removed by sonication only. A cellulase treatment, plus sonication, was required to remove them from the root. The third phase was less-well characterized and may be a composite of the first two phases plus an additional mechanism of attachment. The fourth phase appeared to be non-specific adherence. Attachment was independent of the induction of Nod factors and neither induction by genistein or diadzein, or the use of a *nodA−* mutant showed significant differences compared to wild-type strains. Attachment required macromolecules from both the bacteria and the plant, which exist on both prior to the assay. Using an *in vitro* assay, ^{35}S-labeled *B. japonicum* was able to bind to a ~25 kDa root hair membrane protein separated by SDS-PAGE.

1. Introduction

Nitrogen-fixing symbioses between leguminous plants and rhizobia have become important model systems for the study of plant-microbe interactions. Great strides have been taken toward delineating the early signaling events leading to successful infection of the host plant by its bacterial partner. However, much less is known about the processes by which rhizobia become physically attached to the roots of their respective host legumes. It has been demonstrated that the plant phenolics alone in the absence of the plant are able to induce the formation of rhizobial Nod factors, and furthermore, the Nod factors in the absence of the rhizobia can induce meristematic activity in the root. These results imply that physical contact between the symbionts is not a necessary function to initiate meristematic activity. In the soil the exchange of these chemical signals would be more efficient if the rhizobia and plant were in physical contact with one another.

Generally, studies measuring the attachment of rhizobia to plant roots have been conducted from periods of 10 minutes to several hours. The operational definition of attachment of *Bradyrhizobium japoniucm* to soybean roots was defined as those bacteria that remain bound to the root surface after manual washing, but are removed by either sonication or maceration of the root (Oresnik et al., 1998; Vesper and Bauer, 1985). Vesper and Bauer reported contact times as short as two minutes and found significant numbers of *B. japonicum* had attached to soybean roots (Vesper and Bauer, 1985). Their results demonstrated that *B. japonicum* attached to soybean roots in a linear time-dependent manner from 2 to 60 minutes, after which no further bacteria were attached.

Here we report on the attachment of *B. japonicum* to soybean roots at time intervals as short as a few seconds. The attachment of *B. japonicum* to soybean roots was found to be rapid and occurred in several phases within a time frame of 5 minutes.

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2. Materials and Methods

Bacterial strains

Bradyrhizobium japonicum 2143 has been described previously (Karr and Emerich, 1988). *Escherichia coli* strain DH5α was obtained from J. Wall, Biochemistry Dept., University of Missouri. *B. japonicum* 110 (Cm[r]) and a *nodA*[−] (Kan[r]) mutant derived from 110 were provided by H. Hennecke (Lamb and Hennecke, 1986). Cultures were grown in either Vincent's medium (Vincent, 1970) or Tully's medium (Tully, 1988) with gluconate or arabinose as the defined carbon source.

Seed germination

Soybean (*Glycine max*) seed, cultivar Maverick or Williams 82, were surface sterilized by soaking the seed for 4 minutes in 70% ethanol, rinsing twice with sterile distilled water, soaking seeds for 4 minutes in 1.25% hypochlorite and rinsing with two liters of sterile distilled water. Penicillin G (125 mg/100 seed) and Nystatin (20 mg/100 seed) was added to drained seeds and mixed to uniformly distribute the antibiotics. Seeds are then placed onto water agar (7.5 g/l) containing Penicillin G (125 mg/l) and Nystatin (20 mg/l) and incubated at 32°C for 48 hours (Oehrle et al., 2000). Germinated seeds were then transferred to a hydroponic apparatus with cotelydons suspended above a nutrient solution in which the hypocotyls were immersed. The seedlings were aerated continuously with sterile filtered air for the next 24 hours at room temperature. The nutrient solution consisted of 0.5 mM $CaCl_2$, 0.5 mM potassium phosphate, 0.5 mM Mg_2SO_4 and 0.5 mM NaCl. 0.5 mM MES and 0.5 mM MOPS [MN solution] containing Penicillin G (125 mg/l) and Nystatin (20 mg/l).

Attachment assays

The procedures described here are based on those developed by Vesper and Bauer (1985). Germinated soybeans were removed from the hydroponic apparatus and rinsed extensively with MN before use in attachment assays. To initiate attachment each root was placed into a 3 ml test tube containing 1×10^7 *B. japonicum* in MN. *B. japonicum* cultures were grown to an O.D.$_{630}$ of ~0.2 prior to dilution in MN.

After various time periods from 1 to 600 seconds the seedlings were removed and rinsed quickly twice in MN and then finally in PBS-Tween [8.0 g/l NaCl, 0.2 g/l KH_2PO_4, 2.14 g/l Na_2HPO_4-$7H_2O$, 0.2 g/l KCl and 0.02% Tween 20]. Generally 4 or 5 germinated seedlings were used per time interval. The hypocotyl was removed with a sharp razor blade, cut into ~1 cm sections and placed into 2 mls of PBS-Tween and put on ice until sonication.

Initial experiments showed that only the sections containing the root hairs demonstrated kinetically unique responses and these sections were used for the experiments described here. The segments were sonicated for 3 one-minute periods at 22 Watts with 30 second intervals on ice. Controls showed no rupture of bacteria during this treatment with complete recovery of culturable cells. An aliquot of liquid above the sonicated segments was removed and placed into 1.5 ml microfuge tubes until they could be plated onto Vincent's (1970) agar medium (<2 hrs), but with 5 g/l mannitol, containing 20 µg/ml nystatin and the appropriate antibiotics (Strain 2143: 80 µg/ml rifampicin, 50 µg/ml nalidixic acid; Strain 110: 30 µg/ml chloramphenicol and *nodA*[−]; 50 µg/ml kanamycin).

The root segments were washed 4–5 times after sonication with sterile cellulase buffer [5 mM MES, 10 mM $MgCl_2$, pH 5.7]. An aliquot of the last wash was plated on Vincent's (1970) agar medium described above to ensure no bacteria remained. Two mls of cellulase buffer containing 15 mg/ml of cellulysin [Cal-Biochem] and 15 mg/ml of hemicellulase [Sigma] was added and incubated at room temperature for 2–4 hours. This time interval was optimal for removal of bacteria by this treatment.

After incubation the segments were sonicated as above and aliquots placed in microfuge tubes until plating on Vincent's (1970) agar medium as described above. Colonies were counted after ~10 days of growth at 28°C. Protease treatment of roots was performed with 1,000 units/ml of Proteinase K, or trypsin, for 30 minutes in MN at room temperature.

An *in vitro* attachment assay, based on the procedure of Waligora et al. (1999), was used to detect *B. japonicum* attachment to root hair membrane proteins. Root hairs were isolated by the procedure of Werner and Wolf (1987) and the root hair membrane proteins were separated by SDS-PAGE and transferred onto a PVDF membrane. The filter was then placed in a suspension of ^{35}S-methionine plus ^{35}S-cysteine-labeled *B. japonicum* cells (Karr and Emerich, 1996) in MN and incubated for thirty minutes followed by four washes in MN for 30 minutes each. Autoradiography was used to identify a protein to which *B. japonicum* attached.

3. Results

Bradyrhizobium japonicum 2143 was found to attach to soybean roots in as little as 1 second exposure (Fig. 1). The operational definition of attachment is the same as that used by Oresnik et al. (1998) and Vesper and Bauer (1985) that is, those *B. japonicum* which are not removed by manual washing but require sonication for removal from the root surface. These bacteria were enumerated by growth on agar media. The time course of attachment shown was reproducibly obtained from dozens of replicated experiments. Controls in which no *B. japonicum* was added to the roots displayed no colonies indicating that the colonies observed originated from those bacteria to which the root was exposed.
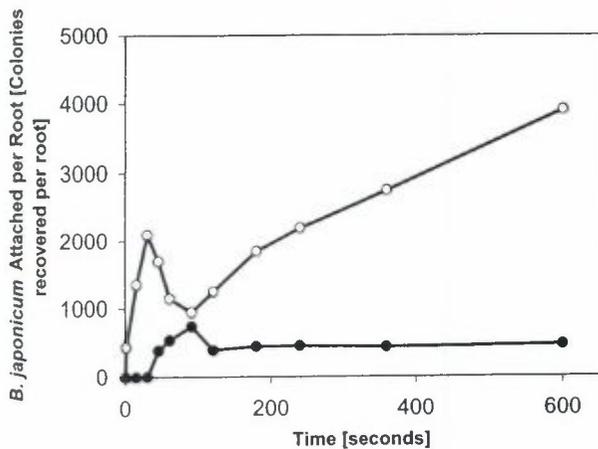
Figure 1. Sonication-removable and cellulase-plus sonication-removable *Bradyrhizobium japonicum* 2143 after attachment to soybean roots for varying time intervals. Soybean roots were placed into *B. japonicum* for varying time intervals as described in Material and Methods. The number of colonies recovered after sonication (O) or after sonication followed by incubation in cellulase followed by another sonication (●) are indicated on a per root basis.
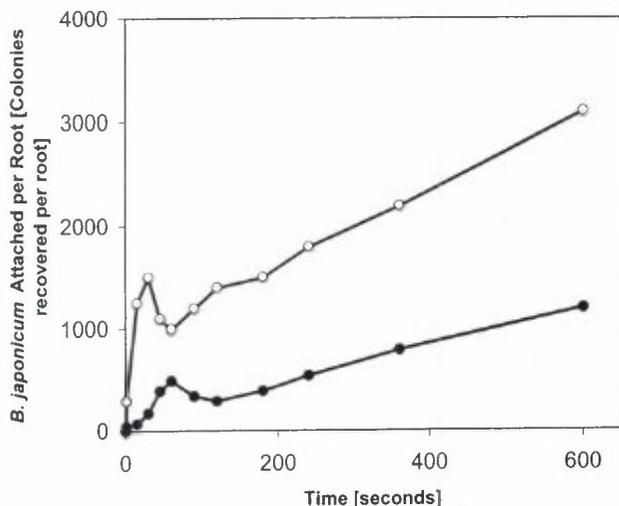

Figure 2. The effect of culture age of *Bradyrhizobium japonicum* 2143 on attachment to soybean roots. Two separate cultures of *B. japonicum* grown to OD_{630} of 0.278 (O) or 1.638 (●) were each diluted to 10^7 bacteria per ml and used in the attachment assays as described in Materials and Methods.

The initial phase of attachment showed a rapid increase in the number of sonication-removable bacteria up to ~30–45 seconds after which a decline in the number of bacteria occurred. This was followed by a third phase of increasing number of bacteria with time but with a slope less than that of the initial phase but greater than that of the fourth and final phase. The third phase was of more variable duration than the other phases and thus was not as discrete and

obvious as the other phases. The observed attachment at each time point, including the one second exposure, resulted in nodulation. There was no difference in the number of nodules per plant and appearance of those nodules with regard to time of exposure (data not shown). The magnitude of all phases depended upon culture age as *B. japonicum* grown to lower cell densities attached in greater numbers than cultures grown to greater cell densities (Fig. 2). The same multi-phasic attachment profile was observed with *B. japonicum* 110 (data not shown).

The attachment of the *B. japonicum* was dependent upon external, surface macromolecules that were removable by sonication. If prior to the assay, *B. japonicum* was sonicated for one minute at the same power setting that was used to remove the bacteria after attachment, it prevented these bacteria from attaching to the soybean roots (data not shown). This sonication treatment did not reduce their survival as judged by the number of colonies on agar plates. The addition of the medium from the sonicated cells, after centrifugation of the *B. japonicum*, did not inhibit attachment by non-sonicated cells. Induction of the bacteria with genistein or diadzein, plant compounds that cause the expression of Nod factors (Kosslak et al., 1987), prior to attachment had no significant effect on either the number of *B. japonicum* bound, or on the attachment time course. Similarly, the number of *B. japonicum* bound was not affected by the organism's inability to synthesize Nod factors. When a *nodA⁻* mutant was used in the attachment assay, the resulting profile was identical to that of the wild-type (data not shown). This implies that Nod factors are not required for attachment of *B. japonicum* to the root surface.

Pre-treatment of the roots with proteinase or trypsin prior to the attachment assay prevented the bacteria from attaching to the root (data not shown). This indicated that attachment of the *B. japonicum* to the roots was dependent upon a protein(s) or a protein-containing macromolecule on the root surface.

After sonication to remove the applied *B. japonicum*, incubation with cellulase and hemicelluase removed additional bacteria from the roots (Fig. 1). The cellulase-dependent release of these cells correlated with phase two, which was when the number of cells removed by sonication only declined. Treatment of *B. japonicum* with cellulase and hemicellulase prior to the attachment assay markedly reduced the decline in colonies recovered after the first sonication, which was a characteristic of phase two, and eliminated the cells removed by the cellulase treatment (data not shown). The number of cellulose-removable cells was generally higher than the number of foci observed on the roots of soybean after prolonged exposure with *B. japonicum*, but was within an order of magnitude (Green and Emerich, 1999).

Phase four was greatly reduced by the inclusion of 100 mM NaCl and 0.02% Tween 20 in the incubation medium. When the slope of phase four was subtracted from the control curve of Fig. 2, the derived values approximated the

values obtained in the presence of the salt and detergent (data not shown). Furthermore, the treatment did not alter the nodulation kinetics or statistically reduce the number of nodules (data not shown). Pre-treating the roots with 10^8 cells/ml of *E. coli*, or with 10^8 cells/ml of *Rhizobium meliloti*, prior to attachment with *B. japonicum* markedly reduced the number bound in phase four.

An *in vitro* assay was used to detect a ~25 kDa membrane protein (monomeric molecular weight) in the root hairs of soybean that binds to *B. japonicum* (Fig. 3). Comparable membrane protein preparations of soybean roots from which the root hairs have been removed or regions of the root that do not have root hairs, do not show binding to *B. japonicum*. The binding of radiolabeled *B. japonicum* could be eliminated by addition of excess unlabeled *B. japonicum*.

4. Discussion

Vesper and Bauer (1985) reported the attachment of *B. japonicum* to soybean roots over the period of 2 minutes to several hours. Over this time period, the attachment was linear for up to 60 minutes. If their data points were extrapolated to the y-axis, labeled as colonies bound per root, at zero time, the extrapolated line would intersect at the positive y-axis. This indicated that there was very rapid binding to the root within the first two minutes of exposure. Because of the relatively large numbers of *B. japonicum* bound to the root in as little as 2 minutes, we decided to investigate shorter time intervals.

The results presented here showed that *B. japonicum* bound to roots very rapidly and in a multi-phasic manner. The multiple phases were reproducible, but the magnitude was dependent upon the age of the culture. The developmental stage of the plant was not investigated here, as 48 hour seedlings were always used, but it may also have an effect. The exposure for only 1 second was sufficient to lead to normal nodulation kinetics and nodule numbers.

The attachment demonstrated here was much more rapid than that believed to occur from previous reports (Green and Emerich, 1999; Matthysse and McMahan, 1998; Michiels et al., 1991; Smit et al., 1992; VandeBroek and Vanderleyden, 1995). The data indicate that there are two distinct phases of attachment plus two additional phases. The first phase apparently requires a sonication-labile macromolecule on the bacteria and a protein(s)-containing macromolecule on the surface of the root. Sonication of the bacteria and protease treatment of the root prevents the first phase of attachment. The second phase appears to be the firmer attachment of *B. japonicum*, presumably via pre-existing cellulose, or polysaccharide macromolecules from the bacterium to the plant. Pre-treatment of the *B. japonicum* with cellulase and hemicellulase prior to attachment, reduced the magnitude of the decline of phase two and eliminated the release of the bacteria in the post-
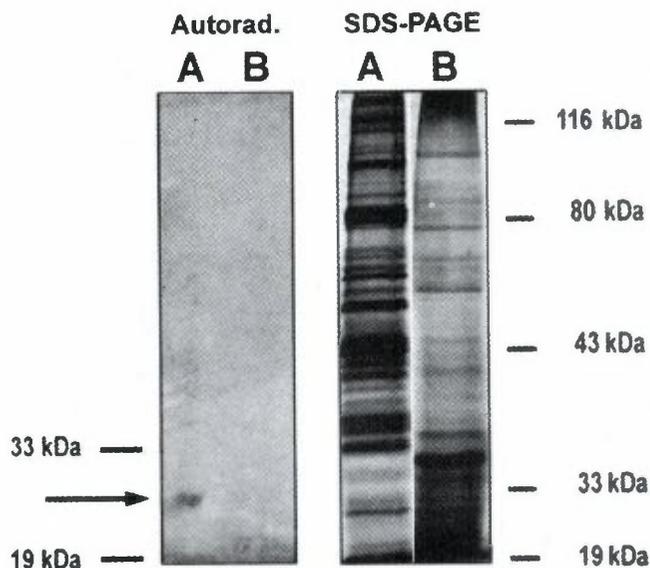

Figure 3. Identification of a soybean root hair membrane protein that binds to *Bradyrhizobium japonicum*. Figure on the left is an autoradiogram of the SDS-PAGE on the right. Lane A of the SDS-PAGE are membrane root hair proteins and Lane B are root membrane proteins minus root hairs. The arrow on the autoradiogram of Lane A indicates the presence of a protein to which ^{35}S-labeled *B. japonicum* cells attached to the protein on the PVDF membrane.

sonication cellulase treatment. The results presented here do not indicate the nature of the third phase, but it may be a non-synchronous combination of phases one and two. The number of cellulase-removable bacteria usually showed a small decline that correlated with phase three suggesting an additional mechanism of attachment to the root. The fourth phase appears to be largely non-specific adherence.

Several phases of attachment have been reported in other microbe plant interactions (Michiels et al., 1991; Smit et al., 1986, 1987, 1992; VandeBroek and Vanderleyden, 1995). Dazzo et al. (1984) reported two phases of attachment by *Rhizobium trifolii* onto clover with the first phase occurring over several minutes and the second requiring several hours. In the two step model of *A. tumefaciens*, the first phase was characterized by removal of the bacteria from the host cell by vortexing of the tissue culture cells or by gentle washing of wound sites on leaves (Matthysse, 1986). In the next step of attachment of *A. tumefaciens* cells to plant surfaces, signals coming from the host plant cells caused the bacteria to elaborate cellulose fibrils. Mutants of *A. tumefaciens* in the cellulose biosynthetic genes are unable to attach to *Arabidopsis thaliana* roots (Matthysse and McMahan, 1998). Cellulose microfibrils have been identified in *Rhizobium leuminosarum* bv *trifolii* (Mateos et al., 1995; Napoli et al., 1975), and in *R. leguminosarum* (Ausmees et al., 1999; Smit et al., 1986, 1987) and have been implicated in

attachment of the rhizobia to plant root hairs. These attachment assays were conducted from 2 hrs to overnight (Ausmees et al., 1999; Napoli et al., 1975; Smit et al., 1986, 1987).

The attachment of cellulose or polysaccharide-containing macromolecules from *B. japonicum* occurs in less than 1 minute, a time not sufficient for the host plant cells to elicit a *de novo* signal. Thus, the attachment of *B. japonicum* to soybean roots occurs much more rapidly than that described for *A. tumefaciens* and much more tightly. The manual washes used here prevented the first phase of attachment from progressing into the second phase. If it did not, exposure times less than 30 seconds should allow progression to phase two, cellulase-removable attachment, since the cellulose or polysaccharide containing macromolecules already exist on the surface of the bacteria. Cellufluor staining also demonstrate the presence of cellulose-containing molecules in cultured *B. japonicum* (data not shown).

However, these washes do not prevent, or retard, nodule development, indicating that the washing procedure only temporarily arrests subsequent phases of attachment and eventual nodulation. This observation may be of significant advantage to study the rapid attachment of *B. japonicum* to soybean over a longer time interval.

The *in vitro* assay identified a component from root hair membranes that could be separated by SDS-PAGE and still retain the ability to bind to ^{35}S-labeled *B. japonicum*. This factor may be the protease labile component of Phase 1.

In summary, *B. japonicum* attaches very rapidly and firmly to soybean roots in a multi-phasic manner. The attachment requires macromolecules from both the bacteria and the plant which exist on both prior to the assay and do not need to be induced by prior exposure to, or the exchange of, chemical signals.

Acknowledgements

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