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Characterization of *Rhizobium* Attachment to Soybean Roots

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Abstract

Soybean roots and root segments excised from the most infectible region of the root were exposed to aqueous suspensions of rhizobia. After rinsing to remove unattached or loosely adhering bacteria, the firmly attached rhizobia were enumerated by phase-contrast microscopy or by plate counting of released bacteria. Culture age significantly affected the firm attachment capability of *R. japonicum* strains 110 and 123, but had little effect on attachment of strains 61A76 and 83. Maximum attachment for strains 110 and 123 occurred at early to mid log phases of growth. *R. japonicum* cells attached firmly to intact roots and root segments at an approximately constant rate for the first 30–40 min of incubation. The net rate of attachment then diminished to essentially zero over the following 30 min. When *Rhizobium japonicum* cells from early log phase cultures of either strain 110 or 61A76 were incubated with a large number of root segments, only small subpopulations of the bacteria adhered to the roots. Approximately 25% of the adhering cells of strain 110 remained attached to the root segments despite vigorous rinsing, whereas the remaining 75% adhered loosely and were removed by rinsing. No evidence for host-specific attachment to soybean roots was obtained. Firm attachment of several strains of heterologous *Rhizobium* species to soybean root hairs and segments was about equal to the attachment of *R. japonicum* 110 over a wide range of bacterial concentrations. Galactose inhibited firm attachment of *R. japonicum* to soybean root hairs and root segments, but N-acetylgalactosamine, a more potent hapten inhibitor of soybean lectin binding, did not, indicating that soybean lectin was probably not responsible for the observed firm attachment of *Rhizobium* cells.

Keywords: *Rhizobium*, soybean, adhesion, root, attachment, host specificity, lectin

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1. Introduction

The attachment (binding, adhesion) of *Rhizobium* cells to the roots of host legumes is likely to be an important step leading to successful nodulation. Infections in soybean are initiated primarily in the zone of emerging root hairs (Calvert et al., 1984; Pueppke, 1983), and hair cells loose their susceptibility to infection within 4 to 6 h after emergence due to acropetal root development (Bhuvaneswari et al., 1983; Bhuvaneswari et al., 1980). Thus, *R. japonicum* cells must quickly establish and then maintain contact with the root surface in the zone of newly emerging hairs for several hours if they are to contribute to successful infection and nodulation. The rate of bacterial attachment and the number of attaching rhizobia may also influence root colonization ability and the competitive advantage of one strain of *Rhizobium* over another.

The binding of host root lectins to specific receptors on *Rhizobium* cell surfaces has been suggested to mediate attachment of the bacteria to the root surface. The concept of lectin-mediated, host-specific attachment of rhizobia has been explored most extensively in clover (Dazzo 1980; Dazzo and Brill, 1977; Dazzo and Hubbell, 1975; Dazzo et al., 1976; Dazzo et al., 1983; Dazzo et al., 1984; Zurkowski, 1980). Other studies have suggested the possibility of host lectin mediated attachment of rhizobia to roots of alfalfa (Paau et al., 1981), pea (Kato et al., 1980; Kato et al., 1981) and soybean (Stacey et al., 1980). Several reports, however, have provided evidence for rapid and substantial attachment of rhizobia to roots of non-host plants where attachment mediated by host lectins could not be expected (Chen and Phillips, 1976; Mills and Bauer, 1985; Pueppke, 1984; Shimshick and Hebert, 1978). The present study describes various characteristics of *Rhizobium* attachment to soybean roots and reexamines the question of attachment specificity.

2. Materials and Methods

Rhizobium cultures

Rhizobium japonicum USDA I-110 ARS (azide, rifampicin, streptomycin resistant) was obtained from L.D. Kuykendall, USDA, Beltsville, MD, strains USDA 123 and 83 from D. Weber, USDA, Beltsville and strain 61A76 from Nitragin Co., Milwaukee, WI. *R. trifolii* TA1 was provided by A. Gibson, C.S.I.R.O, Canberra, Australia, *R. leguminosarum* 92A3 and *R. phaseoli* 127K17 were provided by M. Lamborg and W. Evans, respectively, of the Kettering Laboratory, and *R. meliloti* 1021 was provided by S. Long, Stanford University, Palo Alto, CA. For long term storage, freeze-dried bacteria were maintained in sealed ampules. *R. japonicum* suspensions were stored for

periods of up to 1 month at 10^9 cells/ml in water purified by reverse osmosis and deionization (Crist et al., 1984). Cultures were initiated by placing 0.1 ml of water suspension into 100 ml of yeast extract/mannitol/gluconate medium (YEMG) (Bhuvaneswari et al., 1983).

Soybean seed germination and seedling preparation

Soybean seeds (*Glycine max* (L.) Merr. cv. Williams) were obtained from Dewine and Hamma Seed Co., Yellow Springs, OH, and surface-sterilized as described previously (Bhuvaneswari et al., 1980). Six to eight imbibed seeds with unbroken coats were placed on filter paper wetted with 2 ml of purified water in a 9-cm plastic petri dish. The plates were covered with aluminum foil and placed in a growth cabinet for 48 hr at 28°C day and 26°C night, 70% relative humidity and 14 hr photoperiod. These procedures were carried out aseptically.

Rhizobium attachment assays

Assay manipulations were done under aseptic conditions. All experiments were repeated at least two times with two or three replicate samples per assay and two replicate plates per sample. Seedlings with straight roots 3 to 5 cm long were selected. Roots were cut from the rest of the seedling and the roots assembled with the tips touching a plexiglass block. A cutting tool with three razor blades held at fixed positions was used to obtain consistent 1 cm segments of root. One blade was aligned with the root tip and the other blades were used to cut the root at positions 4 mm and 14 mm above the tip. The 4 mm tip segment was discarded and the next segment, 4 to 14 mm above the tip, was transferred to water for assay. This is the zone where most successful infections and nodules would normally be produced following inoculation of intact seedlings (Bhuvaneswari et al., 1980; Calvert et al., 1984; Pueppke, 1983).

The cultures were diluted with purified water or YEMG to the desired bacterial cell concentrations. Viable cell counts were made for each suspension by plating appropriate dilutions. Sets of 10 roots were added to 80×100 mm Pyrex culture dishes containing 30 ml of bacterial suspension. Suspensions were gently agitated during incubation on a rotary shaker at 50 rpm. After a given length of time, the roots were gently removed from the dish with forceps, drained for a few seconds on filter paper, transferred with forceps to 700 ml of water in a 1 l Wheaton bottle and vigorously shaken for 10 sec to remove unattached bacteria from the roots. The root segments were recovered on a Buchner funnel and placed in a 10 ml test tube containing 2 ml of water plus Tween 40 (one drop in 100 ml water). The tubes were placed in a

holder on the cup-horn unit of a Heat Systems Model 370 sonicator. A power level of 50% for 5 min was used to sonicate the tubes. Aliquots of the suspension of released bacteria were then plated directly or diluted and plated on YEMG agar with a Spiral System Inc. (Cincinnati, OH) Model DU plater. Colonies were counted after one week. In some experiments, root segments exposed to bacteria were rinsed and then transferred to 5 ml of water plus Tween 40 in sterile 7 ml glass homogenizers for hand homogenization of the tissue. Aliquots (200μl) of the homogenates were plated and spread by hand.

A variety of sugars, sugar derivatives, salts and other compounds were examined for their effects on attachment. Aqueous solutions of these were freshly prepared at 2× the desired final concentration and then filter-sterilized. The test compound solution was mixed with an equal volume of the *Rhizobium* suspension in sterile water. The test compound and bacteria were incubated together for 5 min before the root segments were added. In each experiment, one compound, in three or four replicates, was compared to a water control. Cell viability was examined at the end of each experiment.

In order to examine attachment to non-living roots, 1 cm segments were exposed to 2.5% glutaraldehyde for 1 hr, then placed in sterile water for 2 hr to eliminate residual glutaraldehyde and the normal attachment assay carried out. To examine attachment by non-living bacteria, a water suspension of strain 110 ARS was kept overnight in a desiccator with a beaker of chloroform. The suspension, shown to contain no viable cells, was then incubated with excised root segments for 15 min and rinsed prior to observation. Scanning electron microscopy (SEM) was used to compare attachment of dead and living bacteria to the segments of host root. Root segments selected for SEM observation were fixed in 3% glutaraldehyde in 50mM potassium phosphate buffer, pH 7.0, for at least 4 hr. The roots were rinsed 3 times in 50mM potassium phosphate buffer and then 3 times in water for 30 min each. Roots were dehydrated in a 10 step acetone series (10% to 100%), critical point dried in a Polaron drying apparatus (Model E 3000), then sputter-coated with gold and observed in an ISI-40 SEM. Three root segments were examined along their entire length. The bacteria on the visible hemisphere of each segment were enumerated, and this number was extrapolated to the whole segment surface.

In some experiments, phase-contrast microscopy was used to estimate bacterial attachment to root hairs. Early log phase cultures of strain 110 ARS were diluted with an equal volume of filtered Jensen's medium (Vincent, 1970) containing either galactose, N-acetylgalactosamine or N-acetylglucosamine. The bacterial suspensions were incubated for 1 hr with root segments from 5-day-old seedlings grown in plastic pouches as described

(Bhuvaneswari et al., 1980). After incubation, the segments were rinsed in 700 ml of water and suspended in filtered, half-strength, N-free Jensen's medium for microscopic examination. The number of bacteria attached to root hairs 25 to 100μm long in the median optical plane was determined at 400×. Thick root segments were normally crushed beneath the coverglass to enable hairs in the median optical plane to be brought into focus. Sets of 10 to 15 root hairs on at least two roots were examined for each treatment by two independent observers in blind experiments. The observers recorded the length and counted the bacteria directly attached to each hair. The bacteria counted were primarily those visible at the edges of the hairs.

Proportion of R. japonicum cells capable of attaching

The proportion of *Rhizobium* in a population that were capable of attaching to root surfaces was examined by adding 175 to 225 root segments to a 15 ml suspension of approximately 10^4 bacteria/ml in a sterile 125 ml flask and incubating on a rotary shaker at 50 rpm. These experiments were designed to provide all the bacteria with an opportunity to come in contact with a root surface within a reasonably short period of time and to adhere if they were capable of doing so. The size of the non-adhering population was monitored by withdrawing 250μl aliquots of the bacterial suspension at 10 min intervals and plating 3 or 4 replicate portions of each aliquot with the Spiral plater to determine the number remaining in suspension. After 80 min incubation, the roots were removed and replaced with a fresh set of roots. The bacteria free in suspension were then monitored, as described above, for the next 50 min. The number of firmly adhering bacteria was determined by sonication then homogenization of the recovered, rinsed root segments and plating replicate aliquots. The root segments in experiments with these long incubation periods were homogenized after sonication to determine whether a significant fraction of the attached bacteria had become resistant to release by sonication. In all cases, the number of bacteria recovered by the homogenization step was less than 10% of the bacteria recovered by the initial exposure to sonication.

Nodulation assay

Seedlings, 48 hr old, were aseptically transferred to a 9 × 6 cm plastic-mesh grid set on an aluminum pan. The seedling roots were dipped into a known concentration of *R. japonicum* in the pan for 15 min and then washed by vigorously shaking them for 10 sec in 700 ml of sterile water in 1 l Wheaton bottles. Inoculated seedlings were transferred to disposable, non-sterile plastic growth pouches (Northrup King Seed Co., Minneapolis,

MN) which had been prewetted with 10 ml of half-strength, N-free Jensen's plant growth medium. The position of the root tip was marked at the time of pouching (Bhuvaneswari et al., 1980). The number and location of nodules on the primary root above the root tip mark were scored 7 to 9 days after inoculation (Bhuvaneswari et al., 1983).

3. Results

Release of rhizobia by sonic vibration

Bacteria from early log phase cultures of strain 110 ARS diluted to 3×10^7 cells/ml were incubated with replicate sets of 10 excised root segments for 15 min. Loosely adhering and non-adhering bacteria were removed by vigorous shaking of the segments in a large volume of water. Bacteria still adhering to the root surfaces after this rinsing procedure were considered to be firmly attached. The firmly attached bacteria were then released from the root surfaces by sonic vibration or by homogenization in a glass homogenizer as described in Materials and Methods. In two experiments, each with three replicate treatments, an average of $1.27 \pm 0.05 \times 10^4$ bacteria/segment were recovered after exposure to sonic vibration. Homogenization led to recovery of an average of $1.09 \pm 0.12 \times 10^4$ bacteria/segment, indicating that sonic vibration and homogenization were comparably effective and reproducible means of releasing firmly attached bacteria. In separate experiments, it was found that exposure to this level of sonic vibration had no detectable effect on bacterial viability (data not shown).

Bacterial subpopulation capable of attaching

Previously published studies did not determine whether all cells in a given *Rhizobium* culture were equally capable of attaching to root surfaces or whether, alternatively, a distinct subpopulation of the bacteria was responsible for the observed attachment. In order to decide between these alternatives and in order to determine the relative proportions of bacteria capable of loosely and firmly attaching to roots, suspensions of early log phase cultures of *R. japonicum* 110 ARS were incubated with a large number of root segments. The number of bacteria remaining in suspension was monitored throughout the incubation period in order to determine the proportion of cells removed from suspension by either loose or firm association of bacteria with the roots.

As shown in Fig. 1, the number of bacteria remaining in suspension decreased to about 85% of the original number during the first 20 min of incubation and then did not change greatly over the next 60 min. Those bacteria

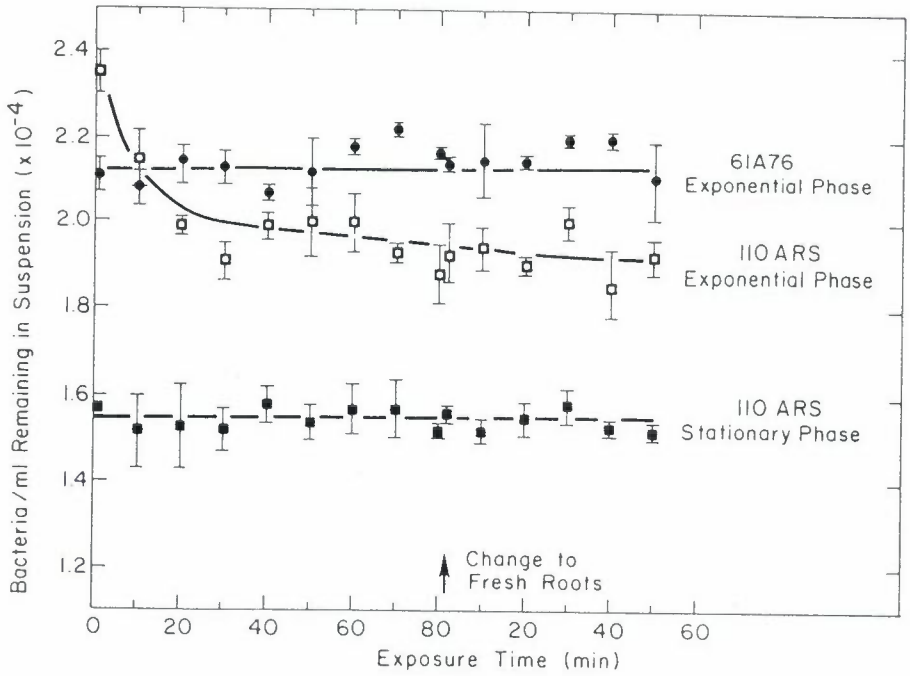

Figure 1. Incubation of *Rhizobium* suspensions with large numbers of soybean root segments. Bacterial suspensions containing approximately 2×10^4 cells/ml were incubated with about 200 root segments. Aliquots of the bacterial suspension were withdrawn at 10 min intervals and plated to determine the number of cells remaining in suspension. After 80 min, the remaining bacteria were incubated with a fresh set of roots and further attachment monitored as before. Each point represents the average from two experiments. The data were analyzed by regression analysis and best fit lines drawn for the exponential phase 61A76 and stationary phase 110 ARS. The data for exponential phase 110 ARS was analyzed by log curve regression analysis and the best fit curve drawn as indicated. The correlation coefficient (R) was 0.919 and the goodness of fit was 0.845.

remaining in suspension after 80 min of incubation with the original set of root segments were subsequently incubated with a fresh set of root segments. Less than 3% of the bacteria from the original suspension attached to the new root segments during a 50 min period (Fig. 1).

The number of firmly attached bacteria recovered by sonication/homogenization was 4.6% of the bacteria in the original suspension or approximately 25% of the total bacteria removed from suspension during the origi-

nal incubation. This indicates that about 75% of the adhering bacteria were loosely associated with the root surfaces, i.e. 15% of the total population in these experiments.

Other experiments with bacteria from stationary phase cultures of strain 110 and from log phase cultures of *R. japonicum* strain 61A76 indicated that the number of unattached cells remained essentially constant for the entire incubation period. (Fig. 1). Thus, the proportion of cells from these cultures capable of either firmly or loosely adhering to soybean roots appears to be smaller than the 5% average standard deviation of sampling. An average of 1.2% of the cells from the stationary phase 110 cultures were found to be firmly attached to the root segments. Approximately 1.3% of the cells from log phase 61A76 cultures were firmly attached. Less than 60 bacteria/segment were recovered by sonication/homogenization of root segments in any of the experiments above.

Time course of Rhizobium attachment

Root segments or intact seedlings were incubated with *R. japonicum* 110 ARS and assayed for firm attachment at intervals over a 2 hr period (Fig. 2). The net rate of firm attachment (i.e. the rate at which the bacteria attached firmly minus the rate at which they detached) was approximately constant during the first 30 to 40 min of incubation, then decreased substantially between 60 and 90 min and was approximately zero by 120 min. There was no appreciable difference between excised root segments and intact roots with respect to the time course of bacterial attachment.

Nodulation of seedlings

Intact seedling roots for nodulation studies were incubated with 10^7 early log phase cells of strain 110 for varying lengths of time, as described in Materials and Methods. The number of nodules which developed in the exposed region of the roots was roughly proportional to the total number of attached bacteria, varying between 1.1×10^3 to 1.8×10^3 firmly attached bacteria per nodule in these experiments (Fig. 2). As a check for attachment to these roots, an average of 2.5×10^3 bacteria/cm were found to firmly attach to 1 cm segments excised from the most infectible region of these roots after 15 min incubation.

Changes in binding characteristics of roots and bacteria

The substantially diminished net rate of firm attachment observed after 60 min of incubation (Fig. 2) could have resulted from either a saturation

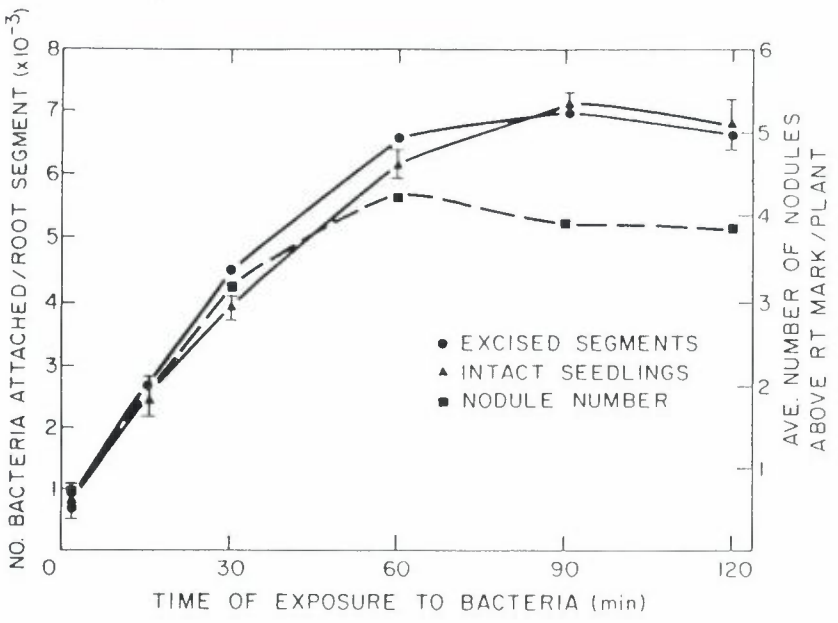

Figure 2. Time course of attachment. Attachment of an early log phase culture of *R. japonicum* 110 ARS to roots was examined over a 2 hr period. Each point shown for the attachment studies is the average from two replicates (10 segments each). Ten segments or 10 seedlings were placed in 30 ml of inoculum on a rotary shaker at 50 rpm. After 15 min, excised segments or intact seedlings were rinsed in 700 ml of water. In the case of intact seedlings, the 1 cm segments were excised after rinsing. The nodulation experiment was performed as described in Materials and Methods. Each data point in the nodulation test is an average from at least 40 plants. Standard deviations are given for the intact seedling attachment.

of suitable binding sites on the roots or from changes in the binding characteristics of either the bacterial cells or the root surface. These possibilities were examined by various treatments initiated after first incubating sets of root segments with aliquots of a bacterial suspension for 60 min. At this time, one replicate set of the suspensions (A) was allowed to incubate an additional 30 min without disturbance. With a second set of the suspensions (B), the "old" root segments were transferred to fresh bacterial suspensions and incubated further for 30 min. The third set of suspensions (C) was incubated for 60 min and then aliquots of the "old" bacterial suspensions were incubated with fresh sets of roots for an additional 30 min. The fourth set of suspensions (D) was incubated for 60 min, shaken vigorously for 10 sec, then

Table 1. Rates of attachment after exposure to fresh roots or bacteria

Treatment	Rate of Attachment as Percent of Control[b]
A) Net rate of attachment for first 30 min (control)[a]	100
B) Old roots and bacteria, undisturbed	0±3
C) Old bacteria + fresh roots	84±4
D) Old roots + fresh bacteria	31±14
E) Old roots and bacteria, briefly shaken	66±31

[a] The net rate of attachment for the first 30 min of incubation (5×10^3 cells/segment) was obtained when 1.5×10^7 110 ARS cells from early log phase cultures were incubated with fresh root segments, as described for Fig. 2.

[b] Mean value ± SD from two experiments with 3 replicates in each

incubated for an additional 30 min. The roots from these various treatments were then assayed for firmly attached bacteria as described in Materials and Methods.

The results of these experiments (Table 1) reveal that about 85% as many "old" bacteria attached to fresh roots over a 30 min period as attached during the first 30 min of the original incubation, suggesting that the bacteria retained almost all of their firm attachment capability during the plateau in net attachment rate. The addition of fresh bacteria enhanced attachment to the "old" root segments relative to the undisturbed control treatment, but the level of such attachment was only about 30% as great as during the original incubation. This result seems to indicate that the root segments changed substantially in their ability to sustain firm bacterial attachment during the original incubation. Brief agitation of the original suspension caused firm attachment of the "old" bacteria to the "old" root segments to increase from essentially zero to approximately two thirds of the original rate.

Effects of culture age on attachment

In an analysis of the effects of culture age on the initial rates of firm attachment, it was found that early to mid-log phase cultures of *R. japonicum* strains 110 ARS and 123 were five to six times greater than attachment of older cultures of these strains and up to ten times greater than attachment of strains 61A76 and 83. Strains 61A76 and 83 showed no detectable culture

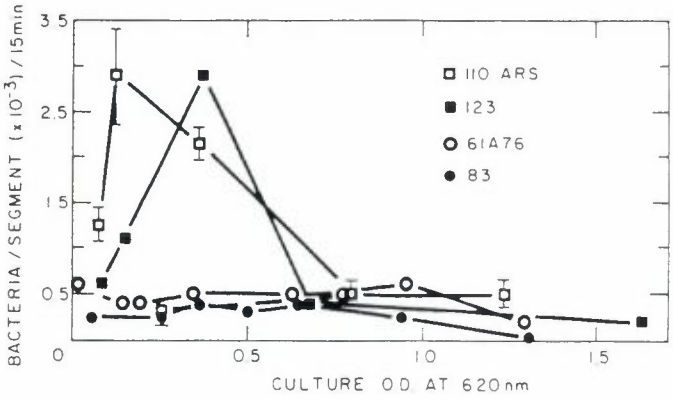

Figure 3. Effects of culture age on attachment of four strains of *R. japonicum* to excised root segments. Cultures of each strain were harvested at the indicated age. Each culture was diluted with YEMG to approximately 10^6 to 10^7 cells/ml and incubated with a set of 10 excised soybean root segments for 15 min. The number of bacteria firmly attached was determined by plate counting as described in Materials and Methods. The number of bacteria attached was normalized to an initial inoculum concentration of 1×10^7 because the initial concentration of bacteria was variable. Actual counts of bacteria attached per segment in 15 min ranged from 5×10^2 to 5×10^3 in these experiments. The results given are for three replicate experiments. Standard deviations are given for strain 110 ARS.

age dependency in their attachment ability (Fig. 3).

Reversibility of Rhizobium attachment to root segments

Sets of 100 root segments were incubated for 15 min with 60 ml of early log phase cultures of 110 ARS (10^7 cells/ml). The root segments were then rinsed three times as described in Materials and Methods and replicate groups of 10 of the rinsed segments were resuspended in water. Subsets of root segments were then assayed at various time intervals to determine the number of bacteria firmly adhering to each set of roots and the number of bacteria released into the suspending liquid from the roots. An average of $3.1 \pm 0.3 \times 10^3$ *R. japonicum* cells adhered/root segment after the 15 min incubation and initial rinsing. During a subsequent incubation in water for 5 min following the initial rinses, an average of 430 ± 60 bacteria/segment came free from the rinsed root segments. When tested at intervals over the next 2 hours, the number of bacteria that remained firmly attached to the root segments was approximately constant, with values ranging from $2.8 \pm 0.15 \times 10^3$ to

$3.3 \pm 0.2 \times 10^3$ per segment. These values do not differ from the original level of attachment ($3.1 \pm 0.3 \times 10^3$) to a statistically significant (T test) extent. Similarly, the number of bacteria recovered in the liquid surrounding the transferred root segments at later times varied from 417 ± 70 to 426 ± 50 per segment, but did not differ significantly from the number (430 ± 60 cells/segment) released during the first 5 min after transfer. Both of these results suggest that only a few of the firmly attached bacteria were released during the 2 hr incubation. Those bacteria which were released from the roots in these experiments were released almost entirely during the first few minutes after transfer and not during the remainder of the 2 hr incubation period.

Concentration dependence of attachment

The effect of *Rhizobium* cell concentration on the number of bacteria firmly attached during a brief 15 min incubation was measured over a range of bacterial concentrations from 10^4 to 10^8 cells/ml. The results (Fig. 4) were analyzed by Michaelis-Menten and Lineweaver-Burk equations as described by Cohen et al. (1981). The Lineweaver-Burk plots appeared to be linear with respect to the dependence of 1/B vs 1/V. The extrapolated Vmax value was 2.2×10^5 bacteria/min/segment and the extrapolated Ks value was 1.1×10^9 bacteria/segment.

Attachment studies with dead roots and bacteria

To determine whether attachment was an active or passive process for the root, root segments were exposed to 2.5% glutaraldehyde and then compared by the 15 min attachment assay to root segments previously exposed to water instead of glutaraldehyde. In two experiments with two replicate samples each, *R. japonicum* 110 ARS adhered equally well to untreated segments as to glutaraldehyde-treated segments ($95 \pm 11\%$ of untreated). On the other hand, attachment of chloroform-killed rhizobia, determined by SEM as described in Materials and Methods, was only $8 \pm 7\%$ of that of untreated rhizobia.

Host specificity

Both *R. trifolii* TA1 and *R. meliloti* 1021 were found to adhere firmly to soybean root segments approximately as well as the homologous species of *Rhizobium* (Fig. 5). The differences were minor over a wide range of concentrations. All cultures for these experiments were in early log phase (OD 0.1 to 0.15 at 620 nm). This culture age was optimal for attachment of 110 ARS (Fig. 3), but not necessarily optimal for the attachment of the heterologous strains.

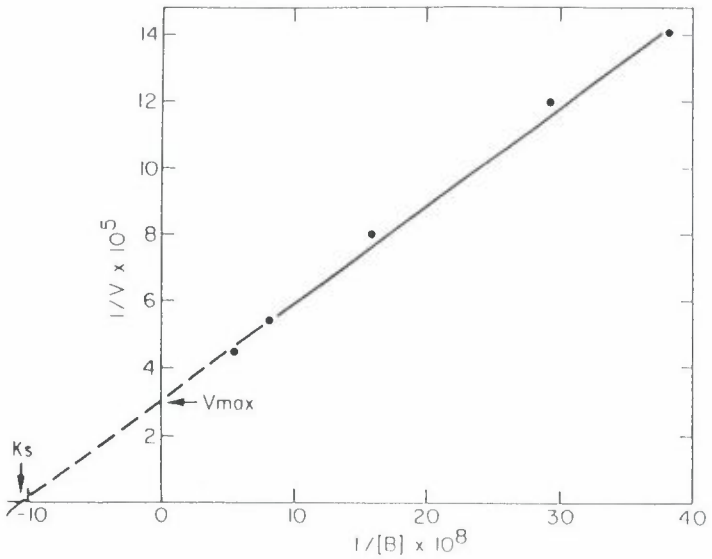

Figure 4. Effect of *Rhizobium* concentration of rates of firm attachment to soybean roots. An early log phase 110 ARS culture was diluted with YEMG to give a series of concentrations ranging from 10^4 to 10^8 cells/ml. Root segments were exposed to each suspension for 15 min and attachment assayed as described in Materials and Methods. B is the concentration of *R. japonicum* 110 ARS cells/ml and V is the number of 110 ARS cells bound to a segment/min of exposure. Data from one of the duplicate experiments was plotted according to the Michaelis-Menton and Lineweaver-Burk equations as described (Cohen et al., 1981).

Phase contrast microscopy was used in further experiments to determine whether rhizobia attached in a host specific manner to the root hairs of soybean roots. *Rhizobium* infections in soybean are primarily, and perhaps exclusively, initiated in young emergent root hairs (Bhuvaneswari et al., 1980; Calvert et al., 1984; Pueppke, 1983; Turgeon and Bauer, 1982; Turgeon and Bauer, 1985). Two additional heterologous strains of rhizobia were compared with *R. japonicum* 110 ARS for attachment to such young emergent hairs as described in Materials and Methods. In two separate experiments with four replicates each, an average of 57 ± 16 bacteria attached per mm of root hair length following incubation with a suspension containing 4×10^7 cells/ml from log phase cultures of strain 110 ARS for 1 hr. Attachment of log phase *R. phaseoli* 127K17 cells (7×10^6 cells/ml) to soybean root hairs under the same conditions averaged 85 ± 28 bacteria per mm of root hair. Bacteria from log phase cultures of *R. leguminosarum* 92A3 (2×10^7 cells/ml) attached

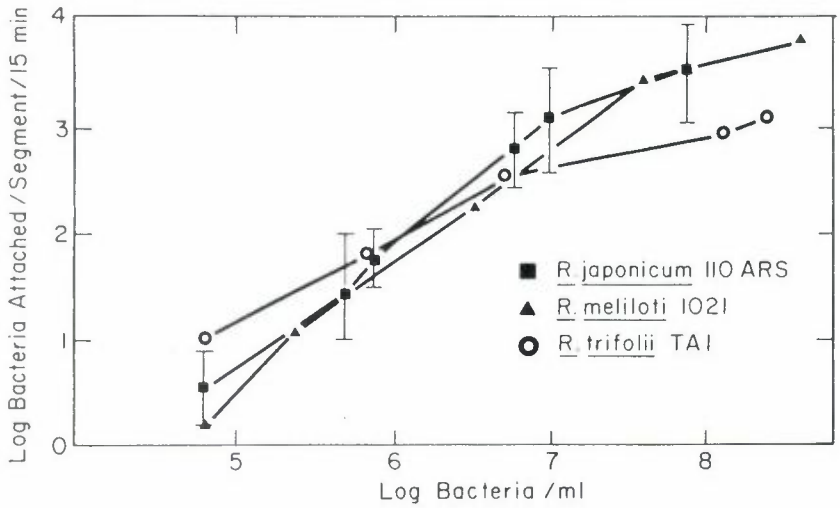

Figure 5. Comparison of attachment of heterologous and homologous *Rhizobium* to soybean root segments. Attachment to soybean roots by *R. japonicum* 110 ARS, *R. meliloti* 1021 and *R. trifolii* TA1 was determined at concentrations ranging from 10^4 to 10^9 cells/ml by the assay described in Materials and Methods. Each species was tested at an early log phase of growth (O.D. 0.1 to 0.15 at 620 nm). Individual data points are averages from three replicate samples from a single experiment for the heterologous strains and combined values from two experiments for strain 110 ARS. Standard error bars are given for attachment of 110 ARS.

profusely to soybean root hairs, often in clusters, and it was difficult to obtain accurate counts. As as estimate, an average of more than 150 bacteria were attached per mm of root hair. Very considerable variations were observed in the number of bacteria attached to individual hairs, regardless of the strain. There was no readily discernible difference between strains with regard to the localization or orientation of attached bacteria on the root hairs.

Effects of sugars and other compounds

In order to examine the possible role of soybean lectin in adherence of rhizobia to the roots, two sugar haptens of soybean lectin binding (galactose and N-acetylgalactosamine) and various non-hapten sugars were tested by the sonic vibration/plate count method for their effects on firm attachment. Of the sugars tested, only galactose inhibited *R. japonicum* attachment to root segments (Table 2). Mannose and N-acetylglucosamine had no discernible

Table 2. Effects of sugars and derivatives on 110 ARS attachment

Bacteria (ranging from 2×10^6 to 8×10^6 cells/ml for plate count assays and 1×10^8 to 3×10^8 for microscopic assays) were exposed to the sugars for 5 min before root segments were added. The roots and bacteria were incubated with the given sugar for 15 min (or 1 hr for microscopic assays), then rinsed and the number of attached bacteria assayed as described in Materials and Methods.

Added substance	Conc. mM	Bacterial attachment	
		Plate counts[a]	Microscopic counts[b]
		(% of Control)	
None	—	100[c]	100[d]
N-acetylgalactosamine	10	115± 29	96± 36
"	30	158± 10	96± 37
D-galactose	10	91± 7	ND
"	30	40± 9	ND
"	60	ND	32± 11
N-acetylglucosamine	30	103± 5	104± 26
D-glucosamine	30	148± 10	ND
D-glucose	30	172± 32	ND
2-deoxy-D-glucose	30	140± 25	ND
Mannose	30	108± 15	ND

[a] Values are the average of three replicates ± SD.

[b] Average from two experiments of the number of bacteria adhering to root hairs/mm of root hair length (± SD).

[c] The control averaged 3.9×10^2 bacteria/root segment.

[d] The control averaged 37 bacteria/mm root hair; (ND = not determined).

effect on adherence. N-acetylgalactosamine, glucosamine, D-glucose and 2-deoxy-D-glucose all promoted firm attachment.

The effects of galactose, N-acetylgalactosamine and N-acetylglucosamine on the attachment of *R. japonicum* to developing root hairs were examined by phase-contrast microscopy (Table 2). The results indicated that galactose partially inhibited attachment to developing root hairs. Neither N-acetylgalactosamine or N-acetylglucosamine were inhibitory.

Potassium nitrate and sodium chloride are known to inhibit nodulation in soybean (Harper and Cooper, 1971; Steinborn and Roughly, 1974). These compounds were tested to learn whether such inhibition might be related to short term effects on bacterial attachment. In a representative experiment with 3 replicates for each treatment, the attachment of strain 110 ARS in a 15 min incubation was enhanced 60 ± 12% by 10mM NaCl and enhanced

$40 \pm 13\%$ by 15mM KNO_3. Casamino acids at a concentration of 6.6 mg/ml also stimulated attachment $42 \pm 3\%$. Attachment was inhibited $44 \pm 12\%$ by the addition of 10mM urea.

4. Discussion

Rhizobium infections in soybean appear to develop almost exclusively in young rather than mature root hairs (Bhuvaneswari et al., 1980; Calvert et al., 1984; Pueppke, 1983; Turgeon and Bauer, 1982; Turgeon and Bauer, 1985). For this reason, our assay methodology normally involved counting just those rhizobia which adhered to the infectible, emergent root hair region of the root. When tested under attachment assay conditions, the number of nodules which formed in the most infectible region of the roots was normal for this strain and cultivar (Bhuvaneswari et al., 1983; Bhuvaneswari et al., 1980) and was proportional to the number of *R. japonicum* cells attached to this portion of the root (Fig. 2). This offered some assurance that the assay method detected and quantitated bacterial attachment which was relevant to subsequent infection and nodule formation. The number of bacteria that attached firmly to excised infectible zone root segments was quite comparable to the number of bacteria that attached firmly to the same region of intact roots (Fig. 2). This indicates that excised root segments could be used in attachment assays if desired without introducing artifacts related to excision.

The present studies help to establish several characteristic features of *Rhizobium* attachment to soybean roots. After incubation of a large number of root segments with a relatively small number of cells from log phase cultures of strain 110 ARS, only about 15% of the bacteria were removed from suspension by association with the roots. Attachment of the bacteria under these conditions was quite rapid and appeared to be largely complete within about 20 min (Fig. 1). The fact that such a small proportion of the bacteria attached under these conditions could be explained if special sites for bacterial attachment on the roots were limited in number and quickly saturated. However, such binding site saturation seems excluded by the fact that approximately 100 times as many bacteria adhered per segment in other experiments (e.g. Figs. 2, 3 and 5). It is also unlikely that any kind of equilibrium was established in these experiments between attached bacteria and free bacteria since the addition of new root segments to the bacteria remaining in suspension did not result in a comparable 15%–20% reduction in the number of free bacteria. We therefore conclude that only a small, distinct subpopulation of bacteria in such cultures of *R. japonicum* is attachment-competent. It follows that the molecular/cellular structures that are needed for adhesion of the bacteria to root surfaces should be present in an effective

form on only a small subpopulation of the cells in a given culture.

From the data in Fig. 1, it appears that *R. japonicum* cells can associate with the host root both firmly and loosely. The firm attachment of bacteria to roots has been emphasized in previously published studies of *Rhizobium* adhesion. It now appears that the majority of adhering bacteria are loosely rather than firmly associated with the roots (Fig. 1). In the future, the methodology used for the experiments presented in Fig. 1 may provide a valuable approach to assessing loose modes of attachment. The role and mechanisms of loose associations certainly appear to merit further study.

Culture age and strain are seen to be important variables in determining the attachment characteristics of *R. japonicum*. Culture age markedly affected bacterial attachment of strains 110 and 123, whereas it had little or no effect on attachment of strains 61A76 or 83. Firm attachment of strain 110 appeared to be maximal at an earlier growth phase than strain 123 (Fig. 3). Substantial differences between various *Rhizobium* strains in their attachment to soybean roots were also described by Pueppke (1984). Further work is required to determine whether the observed effects of growth phase and strain reflect changes in the sizes of the attachment-competent subpopulations, as indicated by the data in Fig. 1, or whether growth phase and strain have an effect on the mechanism(s) by which these bacteria attach.

The existence of attachment-competent and attachment-incompetent *Rhizobium* subpopulations has important implications in terms of ecological adaptation, competition between strains, possible regulatory effects of the host on attachment competence and the isolation of mutants with altered attachment capabilities. The existence of such subpopulations also raises the question of whether attachment-incompetent *Rhizobium* cells are capable of initiating infections.

In other kinds of experiments, the net rate of attachment was found to remain roughly constant throughout the first 30 to 40 min of incubation and then to diminish to approximately zero (Fig. 2). Others have also observed a plateau in the rate of attachment of *Rhizobium* to roots (Badenoch-Jones et al., 1985; Chen and Phillips, 1976; Mills and Bauer, 1985). Mills and Bauer (1985) showed that the diminished rate of *Rhizobium* attachment to clover roots was not due to the saturation of binding sites on the root surface or to the establishment of stronger attachment interactions capable of withstanding sonic vibration. Results in Fig. 2 indicate that the excision of root segments is likewise not responsible for the observed decline in rate of attachment, since the plateau in net rate of attachment was the same for experiments with both intact roots and excised segments.

Our measurements of the kinetics and the extent of bacterial release from roots suggest that the plateau in net rate of firm attachment seen in Fig. 2 does not represent a steady state balance between the rates of attachment and detachment. The rate at which firmly attached bacteria were able to detach from roots was quite low and very nearly zero after the first few minutes of incubation (see Results), whereas bacteria from plateau-phase suspensions could attach to fresh roots at very nearly their original rate (Table 1). Thus, the rate of attachment should be much larger than the rate of detachment and the number of firmly attached bacteria should continue to increase.

The observed plateau in net attachment rate seems to be associated with some change in the characteristics of the roots. We note that vigorous shaking of root/bacterial suspensions for a few seconds increased the observed net rate of firm attachment from essentially zero to close to the original levels (Table 1). This suggests that the plateau may simply reflect a steady state balance between the rate of bacterial attachment to root surface receptors and the rate of release of these receptors, with their associated bacteria, from the root surface. Future characterization of the root surface receptors involved in bacterial attachment will be necessary to address this possibility. Pueppke (1984) has found that attachment of some *Rhizobium* strains to soybean and cowpea roots diminished within an hour, whereas rates for several other combinations remained linear or even increased during a 2 hr incubation. Since these assays were conducted without any agitation during the incubation period, it is possible that bacterial chemotaxis may have played a significant role in determining attachment kinetics in these studies. Whatever the basis for the biphasic time course of the attachment reaction, it seems important that comparisons between strains, cultures or treatments be made during the linear phase of the interaction in order to ensure that important differences will not be obscured by the plateau phenomenon.

A presumably lethal exposure of soybean roots to glutaraldehyde did not affect their ability to adsorb bacteria, whereas killing of the bacteria greatly reduced subsequent attachment. These results suggest that firm attachment requires metabolic activity on the part of the bacteria at some stage of the adhesion interaction, but not on the part of the host.

The concentration dependence of *R. japonicum* attachment to soybean roots (Fig. 4) is very similar to that obtained for *E. coli* attachment to intestinal cell monolayers by Cohen et al. (1981). They suggested that the adhesion of *E. coli* took place in two steps, a reversible step followed by an irreversible step. Their mathematical treatment, based on Michaelis-Menton kinetics, gives Vmax as a measure of the rate of the irreversible binding step. Shimshick and Hebert (1978), on the other hand, suggested that the

attachment of *R. japonicum* to roots of non-leguminous plants was fully reversible and mathematically best modeled by Langmuir isotherms. Our direct measurements of the reversibility of firm *R. japonicum* attachment to soybean roots indicated that 10–15% of the adhering bacteria could detach from roots within the first few minutes after rinsing, but that very few if any adhering bacteria detached spontaneously thereafter during a 2 hr period of incubation. These results appear consistent with the existence of a rapid reversible phase of attachment followed by an essentially irreversible interaction. However, our data are equally consistent with the possibility that some bacterial cells might enter directly into an essentially irreversible association, whereas other bacterial cells might bind reversibly to the roots by an entirely different mechanism.

The partial inhibition of *R. japonicum* attachment by added galactose (Table 2) is consistent with the possibility of bacterial attachment mediated by soybean lectin binding. However, N-acetylgalactosamine is an even more potent hapten inhibitor of soybean lectin binding to *R. japonicum* and red blood cells (Bhuvaneswari et al., 1977; Lis et al., 1970). Therefore, the observed stimulation of bacterial attachment to soybean roots after addition of N-acetylgalactosamine (Table 2) seems inconsistent with attachment mediated by the binding of soybean lectin to both bacterial and root surfaces. It is possible, of course, that soybean roots possess a lectin that differs considerably from the seed lectin in its sensitivity to N-acetylgalactosamine. This remains to be determined.

Evidence presented in Fig. 1 also appears to be inconsistent with the notion that soybean lectin plays a substantial role in either firm or loose attachment of *R. japonicum* to the root surface. Only about 20% of the bacteria from log phase cultures of strain 110 ARS appeared to be capable of attaching to soybean roots (Fig. 1), whereas 50% to 80% of the cells from such cultures are encapsulated and capable of binding substantial amounts of soybean lectin (Bhuvaneswari et al., 1983; Bhuvaneswari et al., 1977).

Careful microscopic analysis of bacterial attachment to soybean root hairs confirmed the lack of inhibition by N-acetylgalactosamine and the partial inhibition of attachment by added galactose (Table 2). In view of these results and other recent studies (Stacey et al., 1984), it seems likely that mechanisms other than host lectin binding are primarily responsible for attachment of *R. japonicum* to soybean roots. Preliminary studies in our laboratory indicate that pili (fimbriae) may play an important role in establishing firm attachment (Vesper and Bauer, unpublished).

Various heterologous strains of rhizobia, strains which are unable to infect

or nodulate soybean, attached to soybean root segments (Fig. 5) and to soybean root hairs just as readily, on the average, as homologous strains. Thus, firm attachment of rhizobia to soybean roots under these conditions was neither host-specific nor host-selective. This result is consistent with the recent observations (Pueppke, 1984) that *Rhizobium* strains attached comparably well to both soybean and cowpea roots regardless of which of these hosts they nodulated.

Stacey et al. (1980) reported that none of the several heterologous *Rhizobium* strains which they examined under similar assay conditions attached at all to root hairs of *Glycine soja*. Further work will be required to establish whether host specificity of attachment is restricted to *Glycine soja* or depends in some fashion on the particular strains or experimental conditions employed in their studies.

The results described by Stacey et al. (1980) support the notion that soybean roots have receptor sites that are suitable for the exclusive attachment of *R. japonicum* cells, whereas our present data and the data of others (Badenoch-Jones et al., 1985; Chen and Phillips, 1976; Van Rensburg and Strijdom, 1982; Mills and Bauer, 1985; Pueppke, 1984; Shimshick and Hebert, 1978) seem to establish that rhizobia can attach comparably well to surfaces of host and non-host roots alike. These views are not mutually exclusive. It is possible that the attachment of some *R. japonicum* cells is mediated by a specific root receptor while at the same time other cells attach non-specifically to the root surface by a different mechanism. Attachment mediated by receptors specific for *R. japonicum* could be qualitatively important even if quantitatively minor if, for example, specific host receptors were localized in places particularly suitable for the initiation of infections, or if binding to a specific host receptor activated responses in the root that were required for infection.

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