APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Apr. 1995, p. 1571–1579 Vol. 61, No. 4
0099-2240/95/$04.00+0

Early Interactions of *Rhizobium leguminosarum* bv. phaseoli and Bean Roots: Specificity in the Process of Adsorption and Its Requirement of Ca^{2+} and Mg^{2+} Ions

ANÍBAL R. LODEIRO, ANTONIO LAGARES, E. NORA MARTÍNEZ,† AND GABRIEL FAVELUKES*

Instituto de Bioquímica y Biología Molecular, Facultad de Ciencias Exactas, Universidad Nacional de La Plata, 1900 La Plata, Argentina

Received 3 October 1994/Accepted 27 January 1995

Roots of *Phaseolus vulgaris* L. were incubated with dilute suspensions (1×10^3 to 3×10^3 bacteria ml^{-1}) of an antibiotic-resistant indicator strain of *Rhizobium leguminosarum* bv. phaseoli in mineral medium and washed four times by a standardized procedure prior to quantitation of adsorption (G. Caetano-Anollés and G. Favelukes, Appl. Environ. Microbiol. 52:371–376, 1986). The population of rhizobia remaining adsorbed on roots after washing was homogeneous, as indicated by the first-order course of its desorption by hydrodynamic shear. Rhizobia were maximally active for adsorption in the early stationary phase of growth. The process leading to adsorption was rapid, without an initial lag, and slowed down after 1 h. Adsorption of the indicator strain at 10^3 bacteria ml^{-1} was inhibited to different extents in the presence of 10^3 to 10^8 antibiotic-sensitive competitor rhizobia ml^{-1}. After a steep rise above 10^4 bacteria ml^{-1}, inhibition by heterologous competitors in the concentration range of 10^5 to 10^7 bacteria ml^{-1} was markedly less than by homologous strains, while at 10^8 bacteria ml^{-1} it approached the high level of inhibition by the latter. At 10^7 bacteria ml^{-1}, all of the heterologous strains tested were consistently less inhibitory than homologous competitors ($P < 0.001$). These differences in competitive behavior indicate that in the process of adsorption of *R. leguminosarum* bv. phaseoli to its host bean roots, different modes of adsorption occur and that some of these modes are specific for the microsymbiont (as previously reported for the alfalfa system [G. Caetano-Anollés and G. Favelukes, Appl. Environ. Microbiol. 52:377–381, 1986]). Moreover, whereas the nonspecific process occurred either in the absence or in the presence of Ca^{2+} and Mg^{2+} ions, expression of specificity was totally dependent on the presence of those cations. *R. leguminosarum* bv. phaseoli bacteria adsorbed in the presence of Ca^{2+} and Mg^{2+} were more resistant to desorption by shear forces than were rhizobia adsorbed in their absence. These results indicate that (i) symbiotic specificity in the *P. vulgaris-R. leguminosarum* bv. phaseoli system is expressed already during the early process of rhizobial adsorption to roots, (ii) Ca^{2+} and Mg^{2+} ions are required by *R. leguminosarum* bv. phaseoli for that specificity, and (iii) those cations cause tighter binding of rhizobia to roots.

Symbiotic associations of the bacterial genera *Rhizobium*, *Bradyrhizobium*, and *Azorhizobium* with their respective leguminous hosts result in nitrogen-fixing nodules and exhibit a remarkable degree of selectivity, implying recognition.

For a long time, this specificity has been thought to occur already in the early stages of the associative process preparatory to root infection. This has been confirmed by the important discovery of the exchange of molecular signals directing the symbiotic process during preinfection: root-exuded flavonoids in the rhizosphere induce soil rhizobia to express *nod* genes and release Nod lipooligosaccharides, and in turn these elicit in the root the process of nodule organogenesis and hair curling (for reviews, see references 10 and 22). Those interactions—especially the latter—take place with a high degree of specificity, which is based on the uniqueness of the respective sets of molecular signals and their fit to the counterpart receptors.

A second type of specific interaction has also been found in the process of preinfection-infection, in which a root lectin or protein participates with symbiotic selectivity by stimulating rhizobia to perform rapid root adsorption and infection (15, 38, 39) and determining the host range of the legume root (11). The mechanisms for these effects are unknown.

Since initial adsorption (synonyms: adhesion, attachment, and binding) of rhizobia to the root surface is one of the early steps in the process of infection preceding root penetration and invasion (18), the possibility that symbiotic recognition might be expressed already at that stage has been raised and repeatedly investigated. Thus, several reports have indicated that some kind of specificity is already expressed during rhizobial adsorption (3, 5, 7, 8, 32) although other evidence has not supported that conclusion (1, 25, 30, 36). This discrepancy could be due, at least in part, to the wide diversity of experimental approaches and conditions (size of rhizobial inoculum per root, root-rhizobium incubation medium, incubation time, washing procedure, and detection, quantitation and location of adsorbed bacteria) used by various investigators to define and study the process of adsorption and its properties. Thus, our laboratory has advocated the use of low numbers of rhizobia in the inoculum (on the order of 10^3 cells ml^{-1} and fewer than 10^4 per root) (2); such dilute inocula are commensurate with rhizobial concentrations that are naturally encountered in soil and are infective for legume roots. This approach has made possible the demonstration of specificity during adsorption of *Rhizobium meliloti* to alfalfa roots (3) and during the interac-

* Corresponding author. Mailing address: Instituto de Bioquímica y Biología Molecular, Facultad de Ciencias Exactas, Universidad Nacional de La Plata, Calles 47 y 115, 1900 La Plata, Argentina. Phone: 54-21-250497, ext. 31. Fax: 54-21-259223. Electronic mail address: fave@qbfelp.edu.ar.

† Present address: Centro de Investigación y Desarrollo en Criotecnología de Alimentos (CONICET-UNLP), Facultad de Ciencias Exactas, Universidad Nacional de La Plata, 1900 La Plata, Argentina.

tion of rhizobia with a protein factor in the alfalfa root exudate which stimulates adsorption to roots (38). On the other hand, the use of such dilute inocula has made the microscopic localization of adsorbed rhizobia on the root surface virtually impossible. In general, the above-mentioned reports have indicated that the early plant-rhizobium interaction process that ends in adsorption is rapid but complex and depends on the physiological state and growth stage of the rhizobia.

The ionic composition of the bacterial growth medium and/or plant-bacterium incubation medium also influences adsorption. It has been shown that both *Rhizobium leguminosarum* bv. viceae (30) and *R. meliloti* (4) require Ca^{2+} and, with lesser efficiency, Mg^{2+} for adsorption to plant roots. Ca^{2+} had been reported long ago as being necessary for nodulation of legumes (21, 26), probably operating at early stages in the development of symbiosis (26). For the *R. meliloti*-alfalfa system, the adsorption step was proposed (4) to be the early Ca^{2+}-dependent event in the pathway to nodulation, postulated by Munns (26) to be inhibited by the absence of Ca^{2+}. However, none of those studies has clarified whether divalent cations do have a role in symbiotic recognition proper.

Adsorption of *R. leguminosarum* bv. phaseoli (in this report, the name *R. leguminosarum* bv. phaseoli [16] is used to designate all rhizobial strains able to form symbiotic nodules in common beans [*Phaseolus vulgaris* L.] without attempting to assign them to any of the currently proposed bean-nodulating species [23, 24, 28]) to roots of its host *P. vulgaris* has been scarcely studied. We present here a characterization of the process of adsorption in this system and explore the existence of symbiosis-specific, as well as nonspecific, modes of the process of adsorption, the particular requirements for Ca^{2+} and Mg^{2+} ions, and the influence of these divalent cations upon the resistance of root-adsorbed rhizobia to desorption from roots.

MATERIALS AND METHODS

Plant material and seed growth. Legume varieties used in this study were common bean var. Dor 41 (black) and Alubia (white) (*P. vulgaris* L.), white clover cv. El Lucero (*Trifolium repens*), and alfalfa cv. Don Arturo (*Medicago sativa*).

Seeds were surface sterilized by immersion in 96% ethanol for 10 s (beans), 15 min (clover), or 30 min (alfalfa) and then in 0.2% $HgCl_2$ in 0.5% HCl for 3 to 5 (beans) or 15 (alfalfa and clover) min; this was followed by six washes with sterile distilled water. Seeds were germinated and grown at 28°C on top of water-agar (1.5%), in darkness, for 3 (beans) or 5 (alfalfa and clover) days.

Rhizobial strains, growth, and maintenance. *R. leguminosarum* bv. phaseoli Rph1002, Rph1003, Rph1004, Rph1005, Rph1006, and Rph1007 were isolated (by A. E. F. Sánchez Caro and G.F.) in virgin terrain in northwest Argentina from spontaneous nodules of wild-growing *P. vulgaris* var. aborigineus Burk. (Baudet) plants (the aboriginal form of *P. vulgaris* originated in the Andean slopes of northwest Argentina and Bolivia). They have been characterized (12a) as type I strains (23), since all of them possess multiple copies of the *nifH* gene, have a host range restricted to common beans, and are unable to induce nodules on *Leucaena leucocephala*. Strain Rph1003-S is a stable, fully symbiotic spontaneous derivative isolated from Rph1003 with resistance to 500 mg of streptomycin $liter^{-1}$.

Other strains used were *R. leguminosarum* bv. phaseoli RphCIAT144 (from Centro de Investigaciones en Agricultura Tropical, Cali, Colombia); *R. leguminosarum* bv. trifolii RtrA118 (spontaneously resistant to 100 mg of trimethoprim $liter^{-1}$), RtrA111, and RtrA113; *R. leguminosarum* bv. viciae RviD54, RviD70, and RviD138 (all from Instituto de Microbiologia y Zoologia Agricolas, Instituto Nacional de Technologia Agropecuaria, Castelar, Argentina); and *R. meliloti* RmeSU47 (streptomycin sensitive) (from E. R. Signer, Cambridge, Mass.), RmeAP8 (from S. M. Lesley, Ottawa, Ontario, Canada), and RmeRf6 (streptomycin sensitive; from B. W. Strijdom, Pretoria, South Africa) and its spontaneous derivative RmeRf6-Spc^r (resistant to 100 mg of spectinomycin $liter^{-1}$), which was obtained in our laboratory.

Rhizobial cells grown in yeast extract-mannitol broth (YMB; mannitol at 10.0 g $liter^{-1}$, NaCl at 0.1 g $liter^{-1}$, $MgSO_4 \cdot 7H_2O$ at 0.2 g $liter^{-1}$, K_2HPO_4 at 0.5 g $liter^{-1}$, yeast extract at 0.4 g $liter^{-1}$) (37) to the late exponential phase were supplemented with 20% (vol/vol) glycerol and stored for up to 1 year at −20°C. Samples of glycerol stocks were cultured and maintained on yeast extract-mannitol-agar (15 g $liter^{-1}$) as working stocks at 4°C.

Rhizobia from working stocks were grown in YMB at 28°C with rotatory shaking at 120 rpm for 72 h, until the stationary phase was reached. These starter cultures were diluted 1:100 in fresh YMB and allowed to resume growth until the late exponential to early stationary phase was reached (2×10^8 cells ml^{-1}; optical density at 500 nm, 0.4 to 0.7, depending on the strain).

All strains were confirmed as positive for nodulation on their respective hosts.

Plant mineral medium. Fåhraeus solution (nitrogen free; $CaCl_2$ at 0.114 g $liter^{-1}$, $MgSO_4 \cdot 7H_2O$ at 0.12 g $liter^{-1}$, KH_2PO_4 at 0.1 g $liter^{-1}$, Na_2HPO_4 at 0.15 g $liter^{-1}$, ferric citrate at 0.005 g $liter^{-1}$ [pH 7.0]; micronutrients omitted) (12) was used for rhizobial suspensions, plant root incubations, and root washes. Where indicated, $CaCl_2$ and/or $MgSO_4$ was omitted; in some experiments, K^+ salts of the respective anions replaced them.

Adsorption experiments. The following antibiotic-resistant rhizobia were used in assays of their adsorption to roots: bean-nodulating Rph1003-S (Str^r), clover-nodulating RtrA118 (Tmp^r), and alfalfa-nodulating RmeRf6-Spc^r (Spc^r).

Adsorption was assayed with the method developed for the *R. meliloti*-alfalfa system (2), with some modifications. Seedlings were incubated in a rhizobial suspension (a late-exponential-to-early-stationary-phase YMB culture of an appropriate antibiotic-resistant labeled strain diluted to $1:10^5$ to approximately 10^3 cells ml^{-1} in Fåhraeus solution; the resulting concentration was measured by dilution and plate counting in triplicate). The numbers of seedlings and incubation volumes, respectively, were as follows: for beans (black beans, var. Dor 41), 10 seedlings and 50 ml; for clover or alfalfa, 15 seedlings and 25 ml. Incubation was done for 2 h (except where noted otherwise) at 28°C with rotary shaking at 50 rpm (2). The seedlings were then taken, cotyledons were excised, rootlets with adsorbed rhizobia were washed four times, each time by shaking with 50 ml of fresh Fåhraeus solution for 1 min at 120 rpm, and the wash fluid was discarded. Washed rootlets were distributed on the bottoms of glass petri dishes and then overlaid with molten (45°C) yeast extract-mannitol agar (supplemented with 50 mg of cycloheximide $liter^{-1}$ and the appropriate antibiotic concentration for selection of the assayed strain). After they were allowed to cool to let the agar set, the rootlets were incubated for 48 h at 28°C. Rhizobia remaining adsorbed on root surfaces were grown in this rich medium and allowed to form microcolonies (controls with uninoculated roots were always negative). Microcolonies were counted from the bottom side of the petri dish only, with a dissecting microscope at a magnification of ×25. The relative degree of adsorption of inoculated rhizobia was expressed as an adsorption index, $\%A$, the percentage of inoculated rhizobia counted as microcolonies on roots (from the bottom side only). Confidence intervals were obtained by proper weighing of the individual data (2).

Adsorption of rhizobia to host roots with competition by other strains was assayed as previously described (3), with a dilute inoculum (approximately 10^3 cells ml^{-1}) of the antibiotic-resistant indicator rhizobial strain as before but in the presence of an antibiotic-sensitive, homologous or heterologous competitor strain (3) at the concentrations indicated in each experiment. Competitors were grown in YMB to the late exponential to early stationary phase and collected in the same way as the indicator strain but centrifuged to remove the culture medium and resuspended in Fåhraeus solution to the final cell density desired. The resulting $\%A$ of the indicator strain under competition, $\%A_c$ (which represents the percentage of adsorption in the presence of very high numbers of competitor strain bacteria), was compared to that of controls without competition ($\%A_o$), and inhibition of adsorption ($\%I$) was calculated as follows: $\%I = 100 \times (\%A_o - \%A_c)/\%A_o$. To estimate the number of *R. meliloti* RmeRf6-Spc^r competitor rhizobia adsorbed to bean roots, after regular washing the excised roots were suspended in the same flask in 50 ml of fresh Fåhraeus medium and sonicated for 10 min in a Branson 1200 sonicator bath containing 200 ml of water (modified from reference 25). The released rhizobia were quantitated by plate counting of serial dilutions of the supernatant. Very few competitor rhizobia remained adsorbed after sonication (counted as microcolonies on roots cultured in yeast extract-mannitol agar supplemented with 100 mg of spectinomycin $liter^{-1}$ as described above), i.e., less than 0.1% of those released by sonication.

The expression "divalent cations" is used in this report to designate the presence in the solution of added mixtures of Ca^{2+} and Mg^{2+} ions (as $CaCl_2$ and $MgSO_4$, respectively) at a 1.8:1 molar ratio. Their actual concentrations are given as the sum of $[Ca^{2+}]$ plus $[Mg^{2+}]$; in the case of Fåhraeus solution, this sum concentration is 0.9 + 0.5 = 1.4 mM. The influence of added divalent cations upon rhizobial adsorption to roots, either without or with competitors, was studied by omitting the addition of $CaCl_2$ and/or $MgSO_4$ to the medium. The contribution of the Mg^{2+} concentration carried over by the growth medium in the diluted inocula from YMB-grown cultures of the indicator strain was estimated to be negligible (less than 10^{-8} M). In other experiments, indicator rhizobia were preincubated (10^6 cells ml^{-1}) in Fåhraeus solution with or without divalent cation salts and after an ionic concentration shift by a $1:10^3$ dilution in the final Fåhraeus type medium (either containing or lacking divalent cations), this rhizobial suspension was incubated with roots to assay for adsorption, either in the absence or in the presence of competitors.

To study the course of desorption of rhizobia by hydrodynamic shear, a set of bean roots to which Rph1003-S rhizobia had been adsorbed during regular rhizobium-plant incubations (in the presence or absence of added Ca^{2+} plus Mg^{2+}) was subjected to 12 consecutive washes instead of the 4 normally used in the routine adsorption assays; each wash was performed exactly as in those assays, by shaking with 50 ml of washing solution for 1 min at 120 rpm. Rhizobia

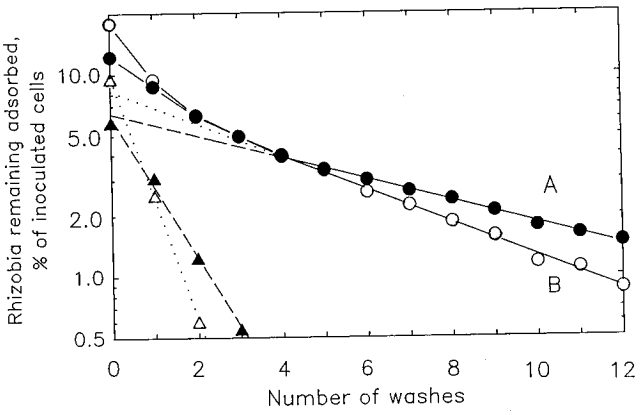

FIG. 1. Course of desorption by repeated washes of strain Rph1003-S adsorbed to bean roots with addition (curve A, filled symbols) or omission (curve B, open symbols) of Ca^{2+} and Mg^{2+} ions. Circles connected by continuous lines represent experimental observations. From washes 4 to 12, a regression line was plotted (curve A, $r = 0.998$; curve B, $r = 0.997$) and projected on the ordinate (curve A, dashed line; curve B, dotted line). These curves represent tightly bound rhizobial populations. To represent loosely bound rhizobial populations, differences between observed values for the percentage of adsorbed rhizobia and theoretical values for tightly bound rhizobia (from regression line projections) were calculated and plotted (filled triangles, rhizobia incubated with added Ca^{2+} plus Mg^{2+}; open triangles, rhizobia incubated without the addition of divalent cations).

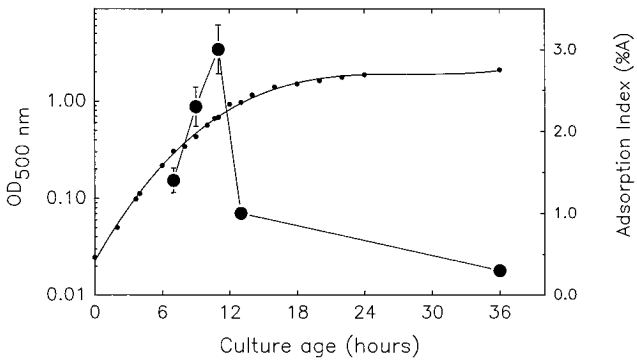

FIG. 2. Influence of the growth phase of strain Rph1003-S on its adsorption to bean roots. Growth was measured as optical density (OD_{500}; small circles). Cultures from different growth stages were diluted to reach the same cell number (approximately 10^3) per milliliter, and the *%A* was determined (large circles). In each experiment, all determinations were done in duplicate. The vertical bars show 95% confidence intervals. Where not represented, the bar is smaller than the symbol. The plot is representative of two independent experiments.

desorbed from roots and contained in each wash were retained on a 0.45-μm-pore-size membrane filter and grown on yeast extract-mannitol agar for plate counts. Rhizobia which remained adsorbed on roots after the 12th wash were counted by the usual procedure of embedding the roots in yeast extract-mannitol agar and growing microcolonies. From these data, the number of rhizobia remaining adsorbed onto the roots after each wash was calculated and log plotted against the wash number.

RESULTS

Characteristics of adsorption of rhizobia to bean roots. In this work (see Materials and Methods and reference 2), adsorption of *R. leguminosarum* bv. phaseoli to common bean roots has been operationally defined by incubating 10 rootlets (whole seedlings) with 50 ml of a dilute suspension of rhizobia (1×10^3 to 3×10^3 bacteria ml^{-1}) in Fåhraeus solution with mild shaking (50 rpm) for 2 h. The seedlings were decanted and washed four times with fresh medium for 1 min each time with shaking at 120 rpm. At that point, root-adsorbed rhizobia were enumerated as described in Materials and Methods to calculate the *%A*.

We studied the course of desorption of rhizobia during many washes. The procedure described in Materials and Methods—originally developed and applied by E.N.M. to the alfalfa system (20)—started with just-incubated and decanted roots prior to any washing, and the numbers of rhizobia remaining adsorbed after each of 12 consecutive standard washes were determined and log plotted against the wash number (Fig. 1, curve A). In this way, a biphasic curve was obtained in which the slope (the log ratio of adsorbed rhizobia remaining on roots after and before the *n*th wash) diminished gradually and attained a constant value after three or four washes. The straight line drawn through washes 4 to 12 had a high regression coefficient ($r = 0.998$) and indicated a first-order dependence of the rate of desorption on the number of adsorbed rhizobia. This behavior was representative of two independent experiments. Thus, after the first two or three washes had carried away rather easily a loosely bound, substantial fraction of the population of originally attached rhizobia, and especially after the fourth wash, there remained a subpopulation of rhizobia which appeared to be homogeneous in the course of their desorption and much more resistant to hydrodynamic shear. Since the procedure for each of the consecutive washes in all of the experiments that followed was exactly the same as that in the above-described study of desorption, we conclude that those rhizobia which remain adsorbed after four washes in the regular assay for adsorption represent the same homogeneous population of firmly adsorbed rhizobia referred to before. With this procedure, we obtained preparations of bean seedlings that had a uniform class of root-adsorbed rhizobia and were devoid of free-living rhizobial precursors present in the original inoculum.

We studied the influence of the growth phase of rhizobial inocula on the process of adsorption. To obtain rhizobial suspensions at different stages of growth, a set of Rph1003-S cultures were started at different times, grown in parallel, and collected simultaneously. Their growth stages ranged from the early log phase to the stationary phase, as indicated by comparison of their optical densities at 500 nm with those of the complete growth curve obtained for this strain in a single culture under the same conditions. Inocula prepared by dilution from each of these cultures and containing similar rhizobial concentrations (1×10^3 to 3×10^3 cells ml^{-1}) were used in assays of adsorption to roots as described in Materials and Methods to determine the *%A* for each inoculum. As shown in Fig. 2, the *%A* of young cultures was very low; it went through a sharp maximum at the early stationary growth phase, decreased with older cultures, and reached nearly 0 at the stationary phase.

In another set of experiments, the time course of the adsorption process was studied (Fig. 3). It started without any apparent lag and slowed gradually after 1 h, tending to a plateau after 4 h: at this time, the *%A* was only double the value determined at 1 h.

Process of rhizobial adsorption to bean roots and symbiotic specificity. The question of whether the symbiotic specificity displayed during infection and nodulation of *P. vulgaris* by *R. leguminosarum* bv. phaseoli is already expressed at earlier stages of their association was next investigated. This was done by following an experimental approach developed before in this laboratory (3), namely, by observing to what extent adsorption of bean-specific indicator strain Rph1003-S (antibiotic resistant; early stationary phase; 10^3 cells ml^{-1}) was inhibited when assayed in the presence of a generally large excess of

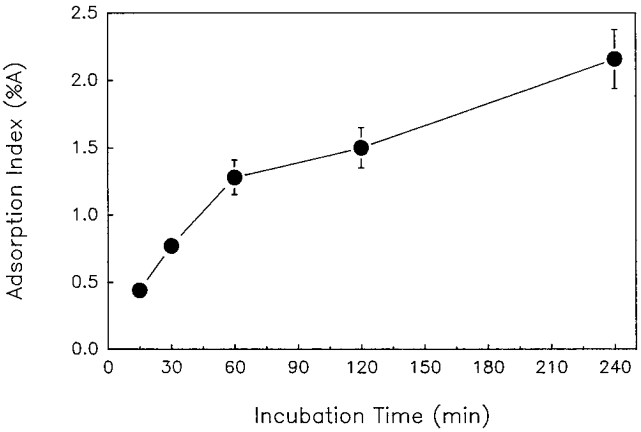

FIG. 3. Time course of adsorption of *R. leguminosarum* bv. phaseoli Rph1003-S to bean roots. In each experiment, all determinations were done in duplicate. The vertical bars show 95% confidence intervals. Where not represented, the bar is smaller than the symbol. This plot is representative of two independent experiments.

other, nonresistant competitor strains (either homologous bean-nodulating strains or heterologous strains belonging to different cross-inoculating groups). In Fig. 4 are shown the results of one of two independent experiments on inhibition of adsorption of the indicator strain *R. leguminosarum* bv. phaseoli Rph1003-S (about 10^3 indicators ml^{-1}), each done in the presence of competitor strain *R. leguminosarum* bv. phaseoli Rph1003 (homologous and isogenic with the indicator strain) or *R. leguminosarum* bv. trifolii RtrA118 (heterologous) in a range of concentrations between 0 and 10^8 competitors ml^{-1}. Roughly S-shaped curves of inhibition of adsorption versus the log concentration of the competitor were obtained, with a very steep rise in inhibition between 10^4 and 10^5 competitors ml^{-1} and smaller increases at higher concentration ranges. A marked difference in the inhibitory abilities of the homologous (isogenic) competitor Rph1003 and the heterologous strain RtrA118 was observed in the upper ranges, from 10^5 to 10^8 cells ml^{-1}, this difference being maximal at 10^7 cells ml^{-1}. In the presence of the homologous competitor, inhibition values tended to increase monotonically approaching 100% at 10^8 competitors ml^{-1}. At variance, through a 100-fold increase of competitor concentrations in the range of 10^5 to 10^7 ml^{-1}, the inhibitory ability of the heterologous strain was limited, the *%I* values being consistently some 20% below those of the homologous competitor. Only at 10^8 cells ml^{-1} did the trend of the curve appear to change and approach the competitive ability of the homologous competitor. This behavior was repeated in the two independent experiments done. A similar inhibitory ability plateau was obtained in the same range of competitor concentrations with the heterologous strain *R. meliloti* RmeRf6-Spc^r, which at 4×10^5, 4×10^6, and 4×10^7 competitors ml^{-1} caused limited inhibitions of 52, 59, and 54%, respectively.

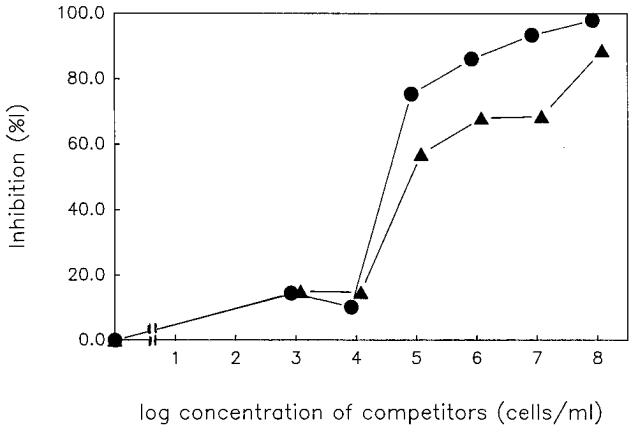

FIG. 4. *%I* of strain Rph1003-S (1.5×10^3 indicator rhizobia ml^{-1}) by the presence of increasing concentrations of homologous (isogenic) competitor strain Rph1003 at 0 to 8.3×10^7 rhizobia ml^{-1} (circles) or heterologous competitor strain RtrA118 at 0 to 1.2×10^8 rhizobia ml^{-1} (triangles). The *%A* of the indicator strain in the absence of competitors was 0.81. This plot is representative of two independent experiments.

In another experiment, we investigated further the inhibitory behavior of competitor strains in the upper range of concentrations tested. Adsorption of the indicator strain Rph1003-S (1.2×10^3 indicators ml^{-1}) was already inhibited 87.1% by 1.6×10^7 cells of Rph1002 ml^{-1} and 83.5% by 1.4×10^7 cells of RphCIAT144 ml^{-1} (both are homologous competitors); with 10-fold higher concentrations of competitors, inhibition values increased to 94.9 and 95.2%, respectively. On the other hand, inhibitions obtained in the presence of heterologous competitors at the 10^7 ml^{-1} level were consistently smaller: in this particular experiment, *%I* was 30.0% with 5×10^7 RmeRf6 cells ml^{-1} and 34.9% with 3.9×10^7 RmeAP8 cells ml^{-1}, whereas upon a 10-fold rise in concentrations, *%I* increased sharply to 87.1 and 79.7%, respectively. As in Fig. 4, these data showed that the limited inhibitory level obtained with a range of 10^5 to 10^7 heterologous competitors ml^{-1} is markedly increased by step raising the concentration of the competitor 1 order of magnitude, to 10^8 ml^{-1}, which now appears to cause high inhibitions, approaching those of homologous competitors. Since differences between the *%I* values obtained in the presence of homologous or heterologous strains were consistently maximal at about 10^7 competitor cells ml^{-1}, this concentration was used in the experiments that followed.

In a search for inhibitory effects in the presence of a broader range of competitor strains, several experiments were performed in which indicator strain Rph1003-S (ca. 10^3 cells ml^{-1}) was incubated in the presence of about 10^7 cells of one of the homologous, bean-nodulating strains Rph1002, Rph1003, Rph1004, Rph1005, Rph1006, Rph1007, or Rph CIAT144, heterologous *R. leguminosarum* bv. trifolii (strain RtrA111, RtrA113, or RtrA118), *R. leguminosarum* bv. viciae (strain RviD54, RviD70, or RviD138), or *R. meliloti* (strain RmeRf6 or RmeSU47) ml^{-1}. The *%I* values observed in individual experiments with the homologous competitors ranged from 82.7 to 92.0% (seven strains in four independent experiments), while the *%I* values obtained with the heterologous competitors were smaller, ranging from 51.0 to 72.0% (eight strains in two independent experiments). Both groups of values were significantly different (*t* test on the arcsin-square root transforms, $P < 0.001$).

These results indicate that—similarly to previous findings obtained with the alfalfa system (3)—during early interactions leading to adsorption of rhizobia to roots of *P. vulgaris*, the specificity of the overall symbiotic association is already expressed.

Influence of divalent cations Ca^{2+} and Mg^{2+} on the process of adsorption of strain Rph1003-S to bean roots in the presence or absence of competitor strains. The preceding results were obtained in experiments in which the mineral medium (N-free Fåhraeus solution) contained regular concentrations of both divalent cations Ca^{2+} and Mg^{2+} (0.9 and 0.5 mM, respectively); these are known to be required and optimal for

TABLE 1. Influence of addition of Ca^{2+} and Mg^{2+} to rhizobium-plant incubation medium on adsorption of different rhizobial species to legume roots[a]

Legume	[Ca^{2+} + Mg^{2+}] added (mM)	%A ± 95% confidence interval		
		R. leguminosarum bv. phaseoli Rph1003-S	*R. leguminosarum* bv. trifolii RtrA118	*R. meliloti* RmeRf6-Spc^r
Phaseolus vulgaris[b]	1.4	1.96 ± 0.20	1.37 ± 0.11	0.44 ± 0.04
	0.0	1.58 ± 0.16	1.04 ± 0.08	0.07 ± 0.01
Trifolium repens[c]	1.4	0.53 ± 0.09	0.59 ± 0.07	0.60 ± 0.07
	0.0	0.33 ± 0.06	0.43 ± 0.06	0.03 ± 0.01
Medicago sativa[c]	1.4	1.25 ± 0.17	0.13 ± 0.03	0.70 ± 0.08
	0.0	1.16 ± 0.15	0.26 ± 0.04	0.07 ± 0.02

[a] Inoculum concentrations, 1.7×10^3 to 3.1×10^3 rhizobia ml^{-1}.
[b] Incubation: 10 seedlings in 50-ml rhizobial suspension.
[c] Incubation: 15 seedlings in 25-ml rhizobial suspension.

adsorption of *R. meliloti* to alfalfa roots (4). We have studied the influence of these cations on the process of adsorption of strain Rph1003-S to roots of *Phaseolus* beans. When assayed in Fåhraeus medium lacking divalent cations (see Materials and Methods), the *%A* in six independent experiments ranged from 79 to 107% of the respective controls with divalent cations, indicating that the process of adsorption does not require the presence of added Ca^{2+} and Mg^{2+}. In a more detailed study, the responses of *R. leguminosarum* bv. phaseoli Rph1003-S, *R. leguminosarum* bv. trifolii RtrA118, and *R. meliloti* RmeRf6-Spc^r to the presence or absence of added divalent cations were compared during adsorption to roots of the respective hosts, Dor 41 beans, white clover, or alfalfa, in all possible homologous and heterologous combinations. The results in Table 1 indicate that in the presence of divalent cations, all three strains showed significant degrees of adsorption to each of the roots (host and nonhost as well). When Ca^{2+} and Mg^{2+} were omitted from the adsorption medium, the *%A* of bean rhizobium Rph1003-S either to roots of its host (81% of control with divalent cations) or to heterologous roots (60 to 93% of controls) was again little affected. Limited effects (73 to 200% of controls) were also obtained under the same conditions for the adsorption of *R. leguminosarum* bv. trifolii RtrA118 to its host, clover, as well as to heterologous roots. In contrast to these results, the ability of *R. meliloti* RmeRf6-Spc^r to adsorb to roots in media lacking divalent cations, not only to its host, alfalfa (as previously reported [4]), but also to heterologous roots, was strongly reduced (5 to 16% of controls).

We also studied whether divalent cations are involved in the expression of specificity in the process of adsorption of bean rhizobia to *P. vulgaris* roots. As shown in Table 2, in the presence of added Ca^{2+} plus Mg^{2+}, specificity was demonstrated as before by the significantly smaller inhibitory effect of heterologous competitor rhizobia at 10^7 bacteria ml^{-1} on adsorption of indicator strain Rph1003-S, compared to the highly competitive effect of homologous, bean-nodulating competitor strains. On the contrary, in the absence of divalent cations, the adsorption of strain Rph1003-S was inhibited by various competitors to generally high degrees. The behavior of heterologous *R. meliloti* competitors was different: inhibition of adsorption was similar and limited with or without added divalent cations. This might be related to the actual numbers of competitor rhizobia bound to roots under those conditions, combined with the previously mentioned existence of a plateau of inhibition (data not shown).

This set of observations indicates that in early interactions participating in the process of adsorption of Rph1003-S to bean roots, the expression of specificity requires divalent cations, while in their absence adsorption of bean rhizobia occurs in other, nonspecific modes which appear to be shared—and competed for—by several heterologous rhizobia.

The level of adsorption of strain Rph1003-S assayed in the presence of a heterologous competitor strain was dependent on the concentrations of divalent cations. Figure 5 shows that when mixtures of Ca^{2+} plus Mg^{2+} ions (molar ratio, 1.8:1) were added in sum concentrations of 0 to 1.4 mM, the *%A* increased and tended to a plateau above 1.0 mM; a half-maximal *%A* was obtained when divalent cations were added in a sum concentration of only 0.15 mM.

In Table 3, experiment 1, the effect on adsorption obtained in the presence of a heterologous competitor strain with the regular mixture of divalent cations (0.9 mM Ca^{2+} plus 0.5 mM Mg^{2+} for a sum concentration of 1.4 mM) was compared with that obtained with either cation added singly at an equivalent concentration. Results suggest that either Ca^{2+} or Mg^{2+} alone could support specificity in the adsorption process of strain Rph1003-S; however, the regular mixture of both appeared to be more efficient. In addition, results shown in Table 3, experiment 2, indicate that the effect obtained with the Ca^{2+} and Mg^{2+} salts could not be attributed to their counteranions ($C1^-$ and SO_4^{2-}, respectively), since these were not stimulatory when present as K^+ salts in the absence of divalent cations.

The effect of divalent cations upon specificity in the process of adsorption in the *Rhizobium*-bean root system might conceivably be exerted through their initial interaction with the rhizobia alone or, on the contrary, with the necessary participation of the root. The former hypothesis is supported by the experiment of Table 4: indicator strain Rph1003-S was preincubated with added 0.9 mM Ca^{2+} plus 0.5 mM Mg^{2+} in the absence of roots and subsequently diluted 1,000-fold with divalent cation-free medium (final concentration, 0.0014 mM); adsorption of the pretreated indicator strain was then assayed in the same medium with addition of roots and a heterologous competitor. In this condition, the diluted divalent cations by themselves would have supported a rather low level of adsorption (cf. Fig. 5 and the results of direct control assays without divalent cations in Table 4). Despite this, pretreatment of the indicator strain was sufficient to confer a high level of ability for subsequent adsorption, equal to that obtained in the control experiment in which rhizobia and roots together were incubated directly in the presence of regular concentrations of divalent cations. This result, which is similar to previous findings obtained with the *R. meliloti*-alfalfa system (4), indicates that the initial target of divalent cations for the effect upon the process of adsorption is solely the rhizobia and not the root.

Influence of divalent cations on the adsorbed state of root-bound rhizobia: resistance to desorption. We have looked for possible differences in the course of desorption of rhizobia which had been adsorbed to roots in media either with or without divalent cations in the absence of competitor strains. In parallel with the experiment of Fig. 1, curve A (done in the presence of Ca^{2+} plus Mg^{2+}), we studied the desorption (by repeated washes) of rhizobia previously adsorbed to bean roots without divalent cations. In this case, the semilog plot of the numbers of rhizobia remaining adsorbed to roots after each consecutive wash as a function of the wash number (Fig. 1, curve B) gave the same type of biphasic curve as curve A. Here again, after rapid desorption during the first four washes, the population of remaining rhizobia desorbed with a much lower constant rate per wash, suggesting a first-order process of desorption. The curves differed in that in the presence of divalent

TABLE 2. Influence of divalent cations on adsorption of *R. leguminosarum* bv. phaseoli Rph1003-S[a] to bean roots with competition by different strains[b]

Competitor strain	Divalent cations added[c]	%A of bean-nodulating indicator strain Rph1003-S ± 95% confidence interval		%I[d]	
		Without competitor (A_o)	With competitor (A_c)	With divalent cations	Without divalent cations
R. leguminosarum bv. phaseoli					
Rph1002	Yes	1.26 ± 0.24	0.22 ± 0.07	82.7	
	No	1.35 ± 0.25	0.07 ± 0.03		94.6
Rph1005	Yes	1.26 ± 0.24	0.10 ± 0.04	91.8	
	No	1.35 ± 0.25	0.06 ± 0.03		95.5
Rph1006	Yes	1.26 ± 0.24	0.17 ± 0.05	86.0	
	No	1.35 ± 0.25	0.05 ± 0.02		96.3
Rph1007	Yes	1.26 ± 0.24	0.14 ± 0.05	88.5	
	No	1.35 ± 0.25	0.11 ± 0.04		91.8
RphCIAT144	Yes	1.07 ± 0.10	0.18 ± 0.03	82.8	
	No	0.85 ± 0.09	0.11 ± 0.02		89.8
R. leguminosarum bv. trifolii					
RtrA118	Yes	1.55 ± 0.17	0.55 ± 0.07	64.6	
	No	1.40 ± 0.15	0.11 ± 0.03		92.3
RtrA111	Yes	1.55 ± 0.17	0.76 ± 0.09	51.0	
	No	1.40 ± 0.15	0.19 ± 0.04		86.0
RtrA113	Yes	1.55 ± 0.17	0.44 ± 0.06	71.4	
	No	1.40 ± 0.15	0.25 ± 0.04		81.7
R. leguminosarum bv. viciae					
RviD54	Yes	1.55 ± 0.17	0.62 ± 0.08	59.8	
	No	1.40 ± 0.15	0.12 ± 0.02		91.2
RviD70	Yes	1.55 ± 0.17	0.66 ± 0.08	57.2	
	No	1.40 ± 0.15	0.13 ± 0.02		90.6
RviD138	Yes	1.55 ± 0.17	0.43 ± 0.06	72.0	
	No	1.40 ± 0.15	0.09 ± 0.01		93.5
R. meliloti					
RmeRf6	Yes	1.07 ± 0.10	0.39 ± 0.05	63.7	
	No	0.85 ± 0.09	0.39 ± 0.05		64.0
RmeSU47	Yes	1.55 ± 0.17	0.63 ± 0.08	59.6	
	No	1.40 ± 0.15	0.60 ± 0.05		57.2

[a] 1.5×10^3 rhizobia ml^{-1}.
[b] 1×10^7 to 2×10^7 rhizobia ml^{-1}.
[c] 0.9 mM Ca^{2+} plus 0.5 mM Mg^{2+} in Fåhraeus solution during rhizobium-plant incubation.
[d] $\%I = [(A_o - A_c) \times A_o^{-1}] \times 100$.

cations (curve A), the linear portion of the slope between the 4th and 12th washes was significantly smaller (12% of adsorbed cells released per wash instead of 18% when divalent cations were absent), indicating greater resistance to desorption. These experiments suggest that the presence of divalent cations during the process of adsorption of strain Rph1003-S to bean roots leads to tighter binding of the rhizobia to root surfaces.

DISCUSSION

Adsorption of rhizobia to their legume host roots, particularly to developing root hair tips, is considered to be one early event in the pathway from free-living roots and bacteria in the soil to infection and nodulation (7, 8, 17, 32, 34, 35). The present study deals with rhizobial adsorption to roots in the *R. leguminosarum* bv. phaseoli-common bean symbiotic system. Our experimental definition of the adsorbed state has two important features, namely, (i) the low concentration of rhizobia in the inoculant suspension and the limited ratio of bacterial numbers per root (around 10^3 cells per ml and fewer than 10^4 per root, respectively) and (ii) the procedure for the root washes. The first feature attempts to reproduce the physiological order of magnitude of rhizobial concentrations frequently encountered in soil by developing legume roots during their

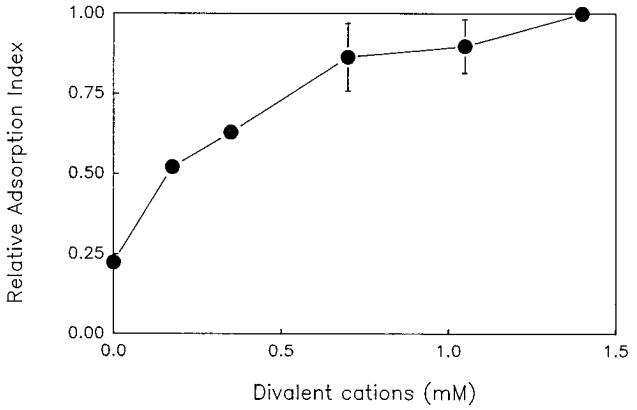

FIG. 5. Dependence of adsorption (*%A* in the presence of a heterologous competitor strain) on the concentration of divalent cations in the rhizobium-plant incubation medium. The relative *%A* is referred to the *%A* reached at 1.4 mM divalent cations—the regular concentration of Fåhraeus solution—as 1.00. Each point represents the mean of two independent experiments. In each experiment, all determinations were carried out in duplicate. The bars represent the standard deviations of the means; where not represented, the value is less than the size of the symbol. The competitor strain used was RtrA118 at a concentration of about 10^7 cells ml^{-1}, giving a competitor-indicator ratio of 10^4:1.

TABLE 4. Effect of preincubation of *R. leguminosarum* bv. phaseoli Rph1003-S with Ca^{2+} plus Mg^{2+} on its adsorption to bean roots with competition by a heterologous strain

Ca^{2+} plus Mg^{2+} concn[a] (mM) during:		*%A* ± 95% confidence interval	
Preincubation[b]	Incubation[c]	Without competitor (A_o)	With competitor (A_c)
	1.40	0.70 ± 0.06	0.30 ± 0.04
	0.00	0.85 ± 0.06	0.08 ± 0.02
0.00	1.40	ND[d]	0.39 ± 0.05
0.00	0.00	ND	0.08 ± 0.03
1.40	1.40	ND	0.42 ± 0.05
1.40	0.0014	ND	0.39 ± 0.05

[a] Sum of concentrations of Ca^{2+} and Mg^{2+} at a molar ratio of 1.8:1.
[b] *R. leguminosarum* bv. phaseoli Rph1003-S (10^6 bacteria ml^{-1}) was preincubated in mineral solution with or without addition of Ca^{2+} plus Mg^{2+} for 90 min at 28°C with rotatory shaking at 50 rpm.
[c] *R. leguminosarum* bv. phaseoli Rph1003-S from the previous treatment was diluted 1:1,000 (resulting concentrations during incubation: 1.4×10^3 to 1.8×10^3 bacteria ml^{-1}) with mineral solution without or with Ca^{2+} plus Mg^{2+} added to the final ionic concentration shown and incubated with 10 bean plants in mineral solution with or without Ca^{2+} and Mg^{2+} for 60 min at 28°C with rotatory shaking at 50 rpm in the presence (where indicated) of competitor strain *R. leguminosarum* bv. trifolii RtrA118 at a concentration of 1.6×10^7 rhizobia ml^{-1}.
[d] ND, not determined.

initial interaction with the free-living microsymbiont. Such a limitation has also been shown to be a necessary condition for the demonstration of specificity during adsorption of *R. meliloti* to alfalfa roots (3) and the participation therein of a protein factor of alfalfa root exudate with specificity (38). The second feature, namely, the specified washing procedure, in our study yielded populations of root-adsorbed rhizobia which appeared to be homogeneous in their associated state, firmly bound to the roots. This was shown with a novel approach by studying the course of desorption of rhizobia from roots subjected to repeated hydrodynamic shear. Kinetic analysis of the results in Fig. 1 indicated the existence of two populations of root-associated rhizobia, one rapidly desorbed, loosely bound, and practically eliminated by the first four washes (as prescribed by our adsorption assay) and the second a firmly attached population which desorbs slowly and homogeneously in a first-order process and represents precisely the rhizobia that remained adsorbed to roots and were counted as such in our assay.

TABLE 3. Influence of ionic composition on adsorption of *R. leguminosarum* bv. phaseoli Rph1003-S[a] and on effects of heterologous competitor strain *R. leguminosarum* bv. trifolii RtrA118[b]

Ion[c] concn (mM) in rhizobium-plant incubation solution				*%A* of *R. leguminosarum* bv. phaseoli Rph1003-S ± 95% confidence interval		*%I*[d]
Ca^{2+}	Cl^-	Mg^{2+}	SO_4^{2-}	Without competitor (A_o)	With competitor (A_c)	
Expt 1						
0.9	1.8	0.5	0.5	1.67 ± 0.20	0.60 ± 0.07	64.1[e]
1.4	2.8	0.0	0.0	1.22 ± 0.19	0.32 ± 0.07	73.8[e]
0.0	0.0	1.4	1.4	1.53 ± 0.24	0.22 ± 0.05	85.6[e]
0.0	0.0	0.0	0.0	1.53 ± 0.24	0.05 ± 0.01	96.7[f]
Expt 2						
0.9	1.8	0.5	0.5	2.34 ± 0.39	1.31 ± 0.22	43.9[e]
0.9	1.8	0.0	0.0	1.42 ± 0.24	0.51 ± 0.11	64.3[e]
0.9	1.8	0.0	0.5	2.13 ± 0.30	0.63 ± 0.13	70.4[e]
0.0	0.0	0.5	0.5	1.88 ± 0.34	0.86 ± 0.15	54.0[e]
0.0	1.8	0.5	0.5	1.96 ± 0.31	0.55 ± 0.11	71.9[e]
0.0	1.8	0.0	0.5	1.88 ± 0.30	0.16 ± 0.02	90.3[f]
0.0	0.0	0.0	0.0	2.30 ± 0.37	0.10 ± 0.01	95.4[f]

[a] Indicator strain concentration, 1.0×10^3 ml^{-1}.
[b] Competitor strain concentrations: 1.7×10^7 ml^{-1} in experiment 1 and 4.2×10^7 ml^{-1} in experiment 2.
[c] Salts used in different media were $CaCl_2$, KCl, $MgSO_4 \cdot 7H_2O$, and K_2SO_4.
[d] $\%I = [(A_o - A_c) \times A_o^{-1}] \times 100$.
[e] Some divalent cation added.
[f] Divalent cations omitted.

As previously found with several rhizobia (*R. leguminosarum* bv. trifolii [9, 29], *B. japonicum* [36], *R. leguminosarum* bv. viceae [19], and *R. meliloti* [38]), the ability of *R. leguminosarum* bv. phaseoli to adsorb to its host root was strongly influenced by the growth state of the bacteria. In the present case, adsorption was maximal in the early stationary phase. The process of adsorption started without an apparent time lag, and its rate diminished gradually after 1 h.

The occurrence of symbiotic specificity during the process of adsorption of rhizobia to their host root hairs preceding infection was demonstrated long ago by microscopic observation in the *R. leguminosarum* bv. trifolii-white clover (7), *B. japonicum*-soybean (32), and in *R. leguminosarum* bv. viceae-pea (17) systems. In the *R. meliloti*-alfalfa system, specificity during adsorption to roots has been demonstrated with a different approach in this laboratory (3).

With this same approach, we studied the expression of specificity in the adsorption of *R. leguminosarum* bv. phaseoli to bean roots. The hypothesis is that when rhizobia become adsorbed to the host root, the involvement of hypothetical, symbiotically specific interactions may be revealed by differential effects that homologous versus heterologous competitors would exert on the adsorption of an indicator, homologous strain.

By measuring rhizobial adsorption in low numbers to roots with competition by an excess of heterologous or homologous rhizobia, we were able to show the occurrence of symbiotic specificity during the process of adsorption of *R. leguminosarum* bv. phaseoli to bean roots. When examined in a range of concentrations from 0 to 10^8 bacteria ml^{-1} (Fig. 4), the competitive ability of the heterologous strain was significantly

lower than that of the homologous competitor, particularly in the interval of 10^5 to 10^7 bacteria ml^{-1} (Fig. 4). When the concentration was increased to 10^8 bacteria ml^{-1}, the inhibition of adsorption by the heterologous competitor tended to approach the value obtained by competition with homologous strains.

On the basis of the isogenicity and presumed equivalence of behavior between the indicator and the homologous competitor in the experiment of Fig. 4, we calculated the total numbers of bacteria (competitor plus indicator) adsorbed to roots and the total rhizobial concentration (sum of both strains) in the inoculum and plotted them in various types of plots. The roughly straight line in the log-log plot (not shown) gave no indication of a phenomenon of saturation in the adsorption of the inoculum; this is similar to findings by Pueppke in the binding of slowly and fast-growing rhizobia to soybean and cowpea roots (27). This agrees with the notion that the process of adsorption comprises a variety of phenomena and rate processes which cannot be described by invoking simple equilibria.

The curves in Fig. 4 suggest that there exist at least two particular types of interaction between the indicator Rph 1003-S and the bean root system, as indicated by the biphasic inhibition curve of heterologous competitor strain RtrA118. The first is represented by the initial portion of the curve extending up to 10^5 bacteria ml^{-1}. The second corresponds to the plateau in the range of 10^5 to 10^7 bacteria ml^{-1} and the increase in competition at 10^8 bacteria ml^{-1}. Comparison with the inhibition curve of the homologous strain shows that a systematic difference in inhibition appears in the region from 10^5 to 10^7 bacteria ml^{-1}. This comprises a residual 20% of the total adsorption, which is exempt from heterologous competition. From the collected findings on levels of inhibition by homologous and heterologous strains, we may infer (with a high degree of statistical confidence) that such interaction complies with the condition of being subject to competition by all homologous strains and immune to all heterologous strains at concentrations of up to 10^7 bacteria ml^{-1} and therefore shows the specificity of the symbiosis.

This conclusion raises the question of the significance of specificity during the process of adsorption for symbiotic associations in general. While there is no direct proof that specificity in adsorption is actually required for the course of preinfection-infection-nodulation, its occurrence in several symbiotic associations has been reported. Besides the previously mentioned cases of white clover (7), soybean (32), pea (17), and alfalfa (3), it has also been observed in common beans (this work). Also, it has been related to the early interaction (with symbiotic specificity) of rhizobia with a lectin or protein factor of the root exudate in several systems: white clover (6, 33), soybean (13, 14), and alfalfa (39). Moreover, this interaction caused increased soybean (14) and alfalfa (39) infectivity and also faster adsorption to alfalfa and clover roots (38).

The preceding experiments were done with rhizobia which had been initially exposed to 0.8 mM Mg^{2+} ions (YMB medium) during bacterial growth and later to the regular concentrations of 0.9 mM Ca^{2+} and 0.5 mM Mg^{2+} ions added during incubation with bean roots. A study of the effects of these cations in this system showed the following. (i) Total adsorption of *R. leguminosarum* bv. phaseoli to roots (in the absence of competitors) did not require addition of divalent cations to the incubation medium with bean roots; this property extended to adsorption to heterologous roots (the same happened with *R. leguminosarum* bv. trifolii but not with *R. meliloti*). (ii) In the presence of heterologous competitors, the process of adsorption of strain Rph1003-S to bean roots required the addition of divalent cations to show specificity: when they were omitted, heterologous strains of *R. leguminosarum* bv. trifolii and viceae were as efficient competitors as homologous strains. On the other hand, the limited competition by heterologous *R. meliloti* against adsorption of the indicator Rph1003-S in the absence of divalent cations (Table 2) appeared to be related to the very reduced adsorption of *R. meliloti* to bean roots (among other legumes) under these conditions. Nevertheless, the number of *R. meliloti* cells actually adsorbed to bean roots without added divalent cations reached a level which, in their presence, would have supported the same limited level of inhibition of adsorption of Rph1003-S.

The full effect of Ca^{2+} plus Mg^{2+} upon specificity in the process of adsorption of *R. leguminosarum* bv. phaseoli to bean roots required a sum concentration of at least 1.0 mM, being half maximal at a 0.15 mM sum concentration (Fig. 5); the simultaneous presence of both ions was necessary for maximal expression of specificity (Table 3). As previously shown for the *R. meliloti*-alfalfa system (4), the effect of divalent cations could be exerted through the bacterial cells, which when preincubated alone with them, gained the ability to adsorb with specificity to bean roots in the near absence of divalent cations (Table 4). This argues in favor of preconditioning of the rhizobia with divalent cations, which is "memorized" by the bacteria to be expressed later.

To investigate whether adsorption performed in the presence or absence of divalent cations led in each case to physically distinct adsorbed states, we measured the resistance of bound rhizobia to hydrodynamic shear. It appeared that in the former condition, adsorbed rhizobia (specifically adsorbed cells comprised among them) were more tightly bound to roots than were bacteria adsorbed in the absence of divalent cations (Fig. 1).

The influence of divalent cations on rhizobial adsorption to roots might involve the participation of rhicadhesin, a protein found in *R. leguminosarum* bv. viceae and proposed to be present also in *R. leguminosarum* bv. phaseoli and other members of the family *Rhizobiaceae* (30). Rhicadhesin is bound to the rhizobial surface by Ca^{2+} (and other divalent cations as well), appears to bridge *R. leguminosarum* bv. viceae to pea root surfaces, and is believed to be responsible for their initial attachment, albeit without specificity (30, 31). In our study, since indicator Rph1003-S rhizobia grown in YMB medium containing a moderate Mg^{2+} concentration (0.8 mM) were not washed prior to their dilution for adsorption, hypothetically associated rhicadhesin molecules might conceivably have stayed bound on rhizobial surfaces and thus could participate in attachment to root surfaces, even in the absence of externally added divalent cations. This would explain the adsorption of *R. leguminosarum* bv. phaseoli to bean roots in divalent cation-free incubations and its lack of specificity. Our results indicate the existence of Ca^{2+}- and Mg^{2+}-dependent modes of adsorption which satisfy the specificity of the symbiotic association and appear to involve other mechanisms besides the mere hypothetical binding by rhicadhesin. Moreover, they point to a novel function of Ca^{2+} and Mg^{2+} ions during the process of adsorption in which—by initial interaction with the rhizobia—these ions participate in the early recognition between symbionts and provide for tighter binding of the rhizobia to roots.

ACKNOWLEDGMENTS

We are indebted to Federico Sánchez, Luis G. Wall, and Daniel H. Grasso for fruitful discussions and encouragement. We also thank Aldo H. Campana for excellent technical assistance.

A.R.L. was supported by CONICET and CICBA, and E.N.M. was supported by UNLP, all from Argentina. A.L. and G.F. are members of the Research Career of CONICET. This work was supported by grants from CONICET and from the Organization of American States.

REFERENCES

1. **Badenoch-Jones, J., D. J. Flanders, and B. G. Rolfe.** 1985. Association of rhizobium strains with roots of *Trifolium repens*. Appl. Environ. Microbiol. **49:**1511–1520.
2. **Caetano-Anollés, G., and G. Favelukes.** 1986. Quantitation of adsorption of rhizobia in low numbers to small legume roots. Appl. Environ. Microbiol. **52:**371–376.
3. **Caetano-Anollés, G., and G. Favelukes.** 1986. Host-symbiont specificity expressed during early adsorption of *Rhizobium meliloti* to the root surface of alfalfa. Appl. Environ. Microbiol. **52:**377–382.
4. **Caetano-Anollés, G., A. Lagares, and G. Favelukes.** 1989. Adsorption of *Rhizobium meliloti* to alfalfa roots: dependence on divalent cations and pH. Plant Soil **117:**67–74.
5. **Dazzo, F. B., and W. J. Brill.** 1979. Bacterial polysaccharide which binds *Rhizobium trifolii* to clover root hairs. J. Bacteriol. **137:**1362–1373.
6. **Dazzo, F. B., and D. H. Hubbell.** 1975. Cross-reactive antigens and lectin as determinants of symbiotic specificity in the *Rhizobium*-clover association. Appl. Microbiol. **30:**1017–1033.
7. **Dazzo, F. B., C. A. Napoli, and D. H. Hubbell.** 1976. Adsorption of bacteria to roots as related to host specificity in the *Rhizobium*-clover symbiosis. Appl. Environ. Microbiol. **32:**166–171.
8. **Dazzo, F. B., G. L. Truchet, J. E. Sherwood, M. E. Hrabak, M. Abe, and S. H. Pankratz.** 1984. Specific phases of root hair attachment in the *Rhizobium trifolii*-clover symbiosis. Appl. Environ. Microbiol. **48:**1140–1150.
9. **Dazzo, F. B., M. R. Urbano, and W. J. Brill.** 1979. Transient appearance of lectin receptors on *Rhizobium trifolii*. Curr. Microbiol. **2:**15–20.
10. **Dénarié, J., F. Debellé, and C. Rosenberg.** 1992. Signaling and host range variation in nodulation. Annu. Rev. Microbiol. **46:**497–531.
11. **Díaz, C. L., L. S. Melchers, P. J. J. Hooykaas, B. J. J. Lugtenberg, and J. W. Kijne.** 1989. Root lectin as a determinant of host-plant specificity in the *Rhizobium*-legume symbiosis. Nature (London) **338:**579–581.
12. **Fåhraeus, G.** 1957. The infection of clover root hairs by nodule bacteria studied by a simple glass slide technique. J. Gen. Microbiol. **16:**374–381.

12a. **González, R. A., A. E. F. Sánchez-Caro, A. R. Lodeiro, G. Favelukes, and O. M. Aguilar.** Unpublished data.

13. **Halverson, L. J., and G. Stacey.** 1985. Host recognition in the *Rhizobium*-soybean symbiosis. Evidence for the involvement of lectin in nodulation. Plant Physiol. **77:**621–625.
14. **Halverson, L. J., and G. Stacey.** 1986. Effect of lectin on nodulation by wild-type *Bradyrhizobium japonicum* and a nodulation-defective mutant. Appl. Environ. Microbiol. **51:**753–760.
15. **Halverson, L. J., and G. Stacey.** 1986. Signal exchange in plant-microbe interactions. Microbiol. Rev. **50:**193–225.
16. **Jordan, D. C.** 1984. Family III. *Rhizobiaceae* Conn 1938, 321, p. 234–256. *In* N. R. Krieg and J. G. Holt (ed.), Bergey's manual of systematic bacteriology, vol. 1. The Williams & Wilkins Co., Baltimore.
17. **Kato, G., Y. Maruyama, and M. Nakamura.** 1980. Role of bacterial polysaccharides in the adsorption process of the *Rhizobium*-pea symbiosis. Agric. Biol. Chem. **44:**2843–2855.
18. **Kijne, J. W.** 1992. The *Rhizobium* infection process, p. 349–398. *In* G. Stacey, R. Burris, and H. J. Evans (ed.), Biological nitrogen fixation. Chapman & Hall, New York.
19. **Kijne, J. W., G. Smit, C. L. Díaz, and B. J. J. Lugtenberg.** 1988. Lectin-enhanced accumulation of manganese-limited *Rhizobium leguminosarum* cells on pea root hair tips. J. Bacteriol. **170:**2994–3000.
20. **Lagares, A., L. G. Wall, E. N. Martínez, and G. Favelukes.** 1988. Early specific recognition between *Rhizobium meliloti* and alfalfa roots, p. 59–60. *In* R. Palacios and D. P. S. Verma (ed.), Molecular genetics of plant-microbe interactions. APS Press, St. Paul, Minn.
21. **Lonergan, J. F., and E. J. Dowling.** 1958. The interaction of calcium and hydrogen ions in the nodulation of subterranean clover. Aust. J. Agric. Res. **9:**464–472.
22. **Long, S. R., and B. J. Staskawicz.** 1993. Prokaryotic plant parasites. Cell **73:**921–935.
23. **Martínez, E., M. A. Pardo, R. Palacios, and M. A. Cevallos.** 1985. Reiteration of nitrogen fixation gene sequences and specificity of *Rhizobium* in nodulation and nitrogen fixation in *Phaseolus vulgaris*. J. Gen. Microbiol. **131:**1779–1786.
24. **Martínez-Romero, E., L. Segovia, F. Martins Mercante, A. A. Franco, P. Graham, and M. A. Pardo.** 1991. *Rhizobium tropici*, a novel species nodulating *Phaseolus vulgaris* L. beans and *Leucaena* sp. trees. Int. J. Syst. Bacteriol. **41:**417–426.
25. **Mills, K. K., and W. D. Bauer.** 1985. Rhizobium attachment to clover roots. J. Cell Sci. Suppl. **2:**333–345.
26. **Munns, D. N.** 1970. Nodulation of *Medicago sativa* in solution culture. V. Calcium and pH requirements during infection. Plant Soil **32:**90–102.
27. **Pueppke, S. G.** 1984. Adsorption of slow- and fast-growing rhizobia to soybean and cowpea roots. Plant Physiol. **75:**924–928.
28. **Segovia, L., P. W. Young, and E. Martínez-Romero.** 1993. Reclassification of American *Rhizobium leguminosarum* biovar phaseoli type I strains as *Rhizobium etli* sp. nov. Int. J. Syst. Bacteriol. **43:**374–377.
29. **Sherwood, J. E., J. M. Vasse, F. B. Dazzo, and G. L. Truchet.** 1984. Development and trifoliin A-binding ability of the capsule of *Rhizobium trifolii*. J. Bacteriol. **159:**145–152.
30. **Smit, G., T. J. J. Logman, M. E. T. I. Boerrigter, J. W. Kijne, and B. J. J. Lugtenberg.** 1989. Purification and partial characterization of the Ca^{2+}-dependent adhesin from *Rhizobium leguminosarum* biovar *viceae*, which mediates the first step of attachment of *Rhizobiaceae* cells to plant root hair tips. J. Bacteriol. **171:**4054–4062.
31. **Smit, G., D. M. J. Tubbing, J. W. Kijne, and B. J. J. Lugtenberg.** 1991. Role of Ca^{2+} in the activity of rhicadhesin from *Rhizobium leguminosarum* biovar *viceae*, which mediates the first step of attachment of *Rhizobiaceae* cells to plant root hair tips. Arch. Microbiol. **155:**278–283.
32. **Stacey, G., A. S. Paau, and W. J. Brill.** 1980. Host recognition in the *Rhizobium*-soybean symbiosis. Plant Physiol. **66:**609–614.
33. **Truchet, G. L., J. E. Sherwood, H. S. Pankratz, and F. B. Dazzo.** 1986. Clover root exudate contains a particulate form of the lectin, trifoliin A, which binds *Rhizobium trifolii*. Physiol. Plant **66:**575–582.
34. **Turgeon, B. G., and W. D. Bauer.** 1982. Early events in the infection of soybean by *Rhizobium japonicum*. Time course and cytology of the initial infection process. Can. J. Bot. **60:**152–161.
35. **Turgeon, B. G., and W. D. Bauer.** 1985. Ultrastructure of infection-thread development during the infection of soybean by *Rhizobium japonicum*. Planta **163:**328–349.
36. **Vesper, J. S., and W. D. Bauer.** 1985. Characterization of *Rhizobium* attachment to soybean roots. Symbiosis **1:**139–162.
37. **Vincent, J. M.** 1970. A manual for the practical study of the root nodule bacteria. IBP handbook no. 15. Blackwell Scientific Publications, Oxford.
38. **Wall, L. G., and G. Favelukes.** 1991. Early recognition in the *Rhizobium meliloti*-alfalfa symbiosis: root exudate factor stimulates root adsorption of homologous rhizobia. J. Bacteriol. **173:**3492–3499.
39. **Wall, L. G., M. C. Giménez, and G. Favelukes.** 1990. Stimulation of *Rhizobium meliloti* for early adsorption and nodulation of alfalfa roots, by previous symbiont-specific interaction with root exudate protein factor or seed agglutinin, p. 279. *In* P. Gresshoff, E. Roth, G. Stacey, and W. E. Newton (ed.), Nitrogen fixation: achievements and objectives. Chapman & Hall, New York.