JOURNAL OF BACTERIOLOGY, June 1991, p. 3492–3499
0021-9193/91/113492-08$02.00/0

Vol. 173, No. 11

Early Recognition in the *Rhizobium meliloti*-Alfalfa Symbiosis: Root Exudate Factor Stimulates Root Adsorption of Homologous Rhizobia†

LUIS G. WALL[1,2] AND GABRIEL FAVELUKES[1]*

Cátedra de Química Biológica I, Facultad de Ciencias Exactas, Universidad Nacional de La Plata, 1900-La Plata, Argentina,[1] and Department of Microbiology, Swedish University of Agricultural Sciences, Uppsala, Sweden[2]

Received 23 July 1990/Accepted 21 March 1991

Adsorption of *Rhizobium meliloti* to alfalfa roots before their infection and nodule formation shows the specificity of the symbiotic association (G. Caetano-Anollés and G. Favelukes, Appl. Environ. Microbiol. 52:377–382, 1986). The time course of specific adsorption of *R. meliloti* (10^3 to 10^4 cells per ml) to roots shows an initial lag period of 3 h, suggesting that either or both symbionts must become conditioned for the adsorption process. Preincubation of *R. meliloti* L5-30 for 3 h with dialyzed alfalfa root exudate (RE) markedly increased early adsorption of rhizobia to alfalfa roots. The activity in RE was linked to a nondialyzable, thermolabile, trypsin-sensitive factor(s), very different from the root-exuded flavonoid compounds also involved in early *Rhizobium*-legume interactions. The lack of activity in the RE from plants grown in 5 mM NO_3^- suggested its negative regulation by the nitrogen nutritional status of the plant. Preincubation of *R. meliloti* with heterologous clover RE did not stimulate adsorption of rhizobial cells to roots. A short pretreatment of RE with homologous (but not heterologous) strains eliminated the stimulatory activity from solution. The stimulation of adsorption of *R. meliloti* to alfalfa roots was strongly dependent on the growth phase of the rhizobia, being greater at the late exponential stage. Nevertheless, the capacity of *R. meliloti* L5-30 to eliminate from solution the stimulatory activity in RE appeared to be constitutive in the rhizobia. The low concentration of rhizobial cells used in these experiments was critical to detect the stimulation of adsorption. The early interaction of spontaneously released alfalfa root macromolecular factor(s) and free-living *R. meliloti*, which shows the specificity and regulatory properties characteristic of infection and nodulation, would be an initial recognition event in the rhizosphere which triggers the process of symbiotic association.

Soil bacteria of the genera *Rhizobium* and *Bradyrhizobium* associate symbiotically with roots of leguminous plants to fix nitrogen. In the process of the symbiotic association, bacteria infect the roots through their root hairs and participate in the joint formation of a specialized root structure, the nodule, within which the rhizobia, differentiated into bacteroids, utilize plant-provided carbon compounds and reduce atmospheric nitrogen for its assimilation by the plant (2, 29).

A remarkable feature of these associations is their high degree of selectivity, whereby only certain pairs of rhizobial strains and legume species are able to form effective nodules. As an example, *Rhizobium meliloti* infects the roots of alfalfa and *R. trifolii* infects the roots of white clover, but crosswise pairings are not compatible.

Starting with the young growing root as it penetrates the soil populated by free-living rhizobia, the early events of the symbiotic process—collectively designated as preinfection—take place with the bacterial cells still outside the root tissues: they comprise rhizobial chemotactic approach to the root, root colonization, attachment to root surfaces, particularly to emerging root hairs, hair deformation and curling, and induction at a distance of a meristem and cortex proliferation at special locations or foci in the root (29, 41).

Some of these events require the participation of the legume root exudate, where a range of substances of root origin—amino acids, sugars, organic acids, flavonoid compounds, polysaccharides, peptides, enzymes, lectins, and other glycoproteins (34)—may influence the behavior of rhizobia. Many of these exudate components are rhizobial chemoattractants (3, 11, 20, 21). In clover, enzymes in the root exudate stimulate polar attachment of *R. trifolii* to surfaces of root hairs (14). In recent years particular attention has been paid to exuded flavonoid compounds. They may play an important role in chemotaxis (7), and they regulate the expression of the *nod* genes in the rhizosphere (30, 32). Macromolecular components of the root exudate, including lectins, also influence the process of infection and nodulation by rhizobia in legume systems such as cowpea (4, 5), soybean (23–25), and clover (36).

It is a generally accepted notion that the recognition between symbionts implicit in their selectivity occurs in the early stages of the associative process. Studies of the nodulation genes of rhizobia (for reviews, see references 29 and 33) have identified genes that control the host specificity of infection in *R. meliloti*. The host range genes have been designated *nodH*, *nodFEG*, and *nodPQ*. These genes, and the regulatory *nodD*, are involved in two types of early specific interactions between symbionts, occurring in sequence. (i) The rhizobial expression of common and host range *nod* genes, which are induced with the participation of the constitutive *nodD* gene product(s) in the presence of flavonoid compounds exuded by the host root, requires the particular set of flavonoids which are characteristic of the homologous host, in a specific interaction with *nodD* (22, 27, 37). *R. meliloti*, carrying three allelic forms of *nodD* (*nodD1*,

* Corresponding author. Electronic mail address: fave%qbfelp.edu.ar@uunet.uu.net.

† Dedicated to the memory of Alberto N. Sarachu.

nodD2, and *nodD3*), is particularly responsive to the alfalfa seed-released flavone luteolin (31) and the root-exuded 4,4′-dihydroxy-2′-methoxychalcone (30). (ii) The symbiotic response of root hair deformation (Had) during preinfection of alfalfa by *R. meliloti* is a result of the specific interaction of roots with an extracellular rhizobial signal (18): a sulfated and acylated glucosamine tetrasaccharide (28) synthesized by the bacteria upon the flavonoid-induced expression of the common *nodABC* and host range *nodH* genes.

Years ago, the introduction of the hypothesis about the role of root lectins in the recognition of the microsymbiont stimulated many studies, which gave contradictory results (13, 26). Recent investigations have confirmed that the root lectin does participate in the process leading to infection and nodulation (16, 25); however, the precise nature of that involvement is not yet known.

Particular attention has also been given to the process of adsorption of rhizobia to the surface of legume roots. Although some authors find that symbiotic specificity is already expressed in this early process (9, 12, 38), others do not (1, 35). However, a comparison of the various results is difficult on account of differences in the approaches used to study rhizobial adsorption to roots.

In this laboratory a method to quantitate adsorption has been developed (8); it allows us to use very dilute bacterial inocula. In this way it was possible to detect a component of symbiotic specificity in the adsorption of *R. meliloti* to alfalfa roots (9).

Here we report studies on the participation of alfalfa root exudate in the process of rhizobial adsorption. The finding of a stimulatory effect of exudate has led to the demonstration of an early rhizospheric interaction between *R. meliloti* and an alfalfa exudate macromolecular, proteinlike factor(s), which precedes and enhances adsorption of rhizobia to roots, and already expresses the specificity of the symbiotic association.

(A preliminary account of these studies was presented to the 7th International Congress on Nitrogen Fixation, Cologne, Federal Republic of Germany, 13 to 20 March 1988; abstract 8-11. These results are part of the doctoral dissertation of L.G.W. presented to the Universidad Nacional de La Plata.)

MATERIALS AND METHODS

***Rhizobium* cultures and plant material.** *R. meliloti* L5-30 (Str^r) (from G. Martínez-Drets, Montevideo, Uruguay), *R. trifolii* A118 (Str^s Trm^r) (from I. Microbiología, INTA-Castelar, Argentina), *R. meliloti* 2011 (from J. Dénarié, Toulouse, France), *R. leguminosarum* 248 (from J. Vasse, Toulouse, France), and *R. phaseoli* CE-3 (from CIFN, Cuernavaca, Mexico) were used in this study. General procedures for the maintenance of stock cultures and for the preparation of starter cultures and inocula were as described before (8). YEM (yeast extract plus mannitol) medium (40) was used.

The alfalfa cultivar Don Arturo (INTA, Anguil, La Pampa, Argentina) and the white clover cultivar El Lucero MA6 (INTA, Pergamino, Buenos Aires, Argentina) were used as sources of root exudate and for studies of bacterial adsorption to root surfaces. Seeds were surface disinfected and germinated on water-agar plates as described before (8).

Except when indicated otherwise, the N-free Fåhraeus solution (FS) (17) was used for plant and bacterial incubations.

Time course of adsorption of *R. meliloti* L5-30 to alfalfa roots. The procedure for adsorption of *R. meliloti* L5-30 to alfalfa roots has been described in detail before (8). Fifteen 5-day-old seedlings were incubated in a rotatory shaker (50 rpm, 28°C) for the indicated time (1 to 12 h) in 22.5 ml of FS containing 10^3 to 10^4 cells of the *R. meliloti* indicator strain per ml (diluted from a late exponential culture [optical density at 500 nm (OD_{500}), 0.3 to 0.4] grown in YEM medium), and roots were washed four times (1 min each) with 25 ml of fresh FS. Root-adsorbed rhizobia were quantitated as described previously (8). In brief, adsorbed bacteria were individually detected and counted along the root surface as microcolonies which developed upon culture of seedlings at 28°C for 2 days embedded in YEM agar, with 100 μg of streptomycin per ml to select for the indicator strain. Adsorption was expressed as adhesiveness (*A*), defined as the percentage of rhizobial cells from the inoculum that remained adsorbed on the root surface (8). Values are given with a 95% confidence interval calculated as described before (8). Specific adsorption of *R. meliloti* to alfalfa roots was measured as indicated above but in the presence of the heterologous competitor *R. trifolii* A118 at a saturating concentration (10^6 to 10^7 cells per ml) (9).

Preparation of RE. Fifty 2-day-old seedlings were aseptically transferred to a stainless steel grid platform in a sterile 250-ml beaker with a glass cover, so that only the roots hanging through the grid remained immersed in 100 ml of FS, and were cultured in a plant growth chamber under controlled conditions (26°C with a 16-h photoperiod) for 3 days. The resulting fluid was the source of crude root exudate. After clarification by centrifugation at 20,000 × *g* for 20 min at 4°C, the supernatant was sterilized by filtration through a polycarbonate membrane (pore size, 0.2 μm; Nuclepore). To eliminate the low-molecular-weight root-exuded substances, we dialyzed the sterilized root exudate (by using membrane tubing with a molecular weight cutoff of 3,500 or 12,000) against FS at 4°C for 20 h, with three changes of fresh FS in a volume ratio of 1/25. The retained material was designated as dialyzed root exudate (RE).

To control contamination during exudation, especially from bacteria endogenously borne by seeds (8), we washed 60 2-day-old seedlings in a rotatory bath (28°C, 100 rpm) for 6 h with 50 ml of FS containing streptomycin (100 μg/ml) and then six times for 1 min each with sterile, fresh FS. The washed plants were used as described above to generate a crude root exudate which was devoid of contaminants as shown by YEM plating.

Preincubation of rhizobia with RE and assay of preincubated bacteria for root adsorption. A rhizobial culture (optical density at 500 nm, 0.3 to 0.4) was diluted as needed with FS, and an aliquot of 2.5 ml was mixed with 10 ml of either RE (experimental) or FS (control) and preincubated in a rotatory bath (28°C, 50 rpm) for 3 h. Afterwards, the experimental incubation mixture was diluted with 12.5 ml of FS and the control was mixed with 2.5 ml of FS plus 10 ml of RE, so that both had the same final composition (containing RE, FS, and approximately 5×10^3 bacteria per ml); where indicated, the control was diluted with only 12.5 ml of FS so that RE was absent. Samples (2.5 ml) of each final suspension were removed for a plate count of the actual number of rhizobia. Fifteen 5-day-old seedlings were immediately immersed, and the suspensions of preincubated rhizobia and plants were then incubated for 1 h in a rotatory bath (28°C, 50 rpm); rhizobial adsorption to roots was measured as described above.

The stimulation of adhesiveness (*S*) caused by the preincubation of rhizobia with RE was expressed as the percent

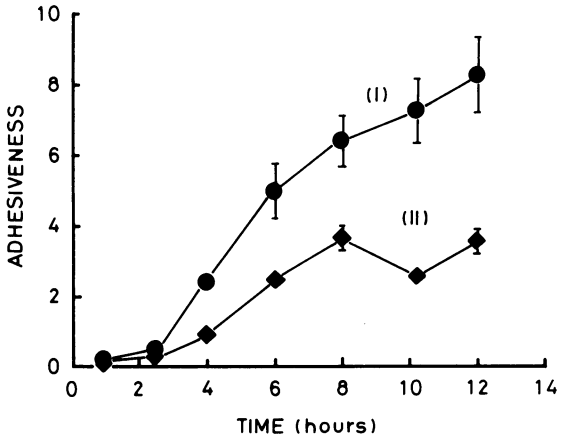

FIG. 1. Time course of adsorption of *R. meliloti* L5-30 to alfalfa roots. Fifteen 5-day-old seedlings were incubated at 28°C for the indicated times with a bacterial suspension from a late exponential culture diluted with FS to 10^3 to 10^4 cells per ml. Rhizobial adsorption is expressed as adhesiveness, the percentage of *R. meliloti* L5-30 bacteria in the initial suspension that remained adsorbed to the roots after specified washing (8). Curve I shows total adsorption in the absence of competitor bacteria. Curve II shows specific adsorption, measured in the presence of *R. trifolii* A118 heterologous competitor bacteria (approximately 10^7 cells per ml) (9). Vertical bars indicate 95% confidence intervals.

increase of adhesiveness $S = (A_P - A_0) \times 100/A_0$, where A_P is the adhesiveness for RE-preincubated rhizobia and A_0 is the adhesiveness of control, FS-preincubated rhizobia.

Treatment of RE with trypsin. Trypsin (type I, from bovine pancreas; Sigma) was added (50 μg/ml) to dialyzed RE and incubated at 28°C for 1 h. Afterwards, soybean trypsin inhibitor (type I-S; Sigma) was added (20 μg/ml) to block any further trypsin activity in treated RE. Trypsin activity was measured by the hydrolysis of *N*-α-benzoyl-DL-arginine-*p*-nitroanilide (Sigma) as a chromogenic substrate. No trypsin activity was detected after addition of the inhibitor.

Pretreatment of RE with rhizobia. A late exponential culture of the appropriate rhizobial strain was centrifuged at 10,000 × *g* for 10 min at 4°C. The pellet was resuspended in alfalfa RE to a concentration of 10^7 cells per ml (or as indicated). This suspension was kept at 28°C for 30 to 40 min without stirring. After that, it was centrifuged at 20,000 × *g* for 20 min at 4°C, and the supernatant was sterilized by filtration (polycarbonate membrane; pore size, 0.2 μm). The filtered supernatant was designated pretreated RE and was assayed for its remaining stimulatory activity by the above-described procedure.

RESULTS

Time course of adsorption of *R. meliloti* L5-30 to alfalfa roots. The adsorption of *R. meliloti* L5-30 to alfalfa roots, which is time dependent (10), was found to vary nonlinearly with the time of incubation of the rhizobia with the plants. Three distinct stages were observed in the course of adsorption (Fig. 1, curve I): an initial lag period of approximately 3 h; a second period when adsorption increased rapidly, faster than the increase in the number of free bacteria in suspension undergoing multiplication; and finally, at about 6 to 8 hours of incubation, a reduction in the increase of adsorbed rhizobia with multiplication continuing at the same rate. This pattern was also observed (Fig. 1, curve II) when specific adsorption of *R. meliloti* L5-30 was assayed in the presence of saturating concentrations (10^6 to 10^7 cells per ml) of the heterologous competitor *R. trifolii* A118 (9). All along the incubation span, the generation time for *R. meliloti* L5-30 in the incubation mixture was 2.5 h, irrespective of the presence of *R. trifolii* A118 at a 10^3-fold-higher concentration (data not shown).

TABLE 1. Effect of preincubation of *R. meliloti* L5-30 with dialyzed alfalfa RE on the subsequent adsorption of rhizobia to alfalfa roots

Preincubation medium[a]	Diluent for incubation with roots[b]	Adhesiveness[c]	Stimulation, %[d]
FS	FS	0.269 ± 0.009 (13)	
FS	RE	0.258 ± 0.007 (26)	
RE	FS	0.470 ± 0.012 (26)	+94 ± 14

[a] *R. meliloti* L5-30 late exponential cultures in YEM medium were diluted to approximately 5 × 10^3 bacteria per ml with FS (lines 1 and 2) or RE (line 3) and preincubated for 3 h.

[b] Each preincubated mixture was diluted with an equal volume of either FS or RE as indicated (see Materials and Methods), and the resulting inocula of preincubated rhizobia were immediately assayed for adsorption to alfalfa roots.

[c] Values are weighted averages of results from independent experiments (numbers of which are indicated in parentheses). Results are given with 95% confidence intervals.

[d] Percent increase of adhesiveness in line 3, compared with the respective control in line 2 in each individual experiment (see the text). The given value is the average of individual percent increases from 26 independent experiments, with 95% confidence interval.

The initial lag period suggested some sort of slow conditioning or preparation of either or both symbionts which increased their readiness for the process of adsorption. Preliminary attempts to perform such conditioning of either the alfalfa roots or *R. meliloti* alone were unsuccessful. Thus, preincubation of the roots alone in FS for increasing periods up to 4 h failed to accelerate adsorption in the subsequent assay; in fact, on the contrary, it tended to decrease it slightly. Moreover, results obtained in this laboratory (27a) suggested that root products that are gradually exuded into the mineral solution and discarded with it are actually required for active adsorption. On the other hand, a decrease of approximately 40% in adsorption (instead of a stimulation) was obtained in a similar experiment in which the rhizobia alone were preincubated in FS for 3 h prior to addition of roots (42). These results have suggested that the development of the hypothetical conditioning effect would require the simultaneous presence of the root exudate and the rhizobia, interacting in a slow process.

Effect of preincubation of *R. meliloti* L5-30 with alfalfa RE on its subsequent adsorption to roots. To detect such a possible participation of root exudate at a very early interaction with rhizobia—prior to a physical contact of these with root surfaces—we studied the effect of preincubating *R. meliloti* L5-30 at low concentration (10^3 to 10^4 cells per ml) with dialyzed alfalfa RE for 3 h, immediately before the 1-h assay for rhizobial adsorption to roots. This procedure attempted to provide an extended period of interaction between the rhizobia and the externally supplied RE (comparable to the time lag of adsorption without preincubation [Fig. 1]), while minimizing any effects of the RE endogenously generated by the roots during the subsequent 1-h adsorption assay. The results of such experiments are shown in Table 1. The preincubation of *R. meliloti* L5-30 with RE resulted in a significant increase (near doubling) of the

ensuing adsorption to roots (line 3), in comparison with the same rhizobial cells which had been preincubated with only FS and then assayed for adsorption either in the presence of newly added RE (line 2) or without it (line 1). Similar results were obtained in preliminary experiments in which the root exudate had not been dialyzed (see below). Since results of adsorption are expressed in terms of adhesiveness (the percentage of rhizobial cells, present at the start of the incubation with the roots, that had been adsorbed onto root surfaces by the end of the assay [8]), the calculated results have taken into consideration any eventual changes in rhizobial numbers occurring during their preceding preincubation with RE.

The stimulation of rhizobial adsorption to roots appeared to require an extended preincubation of rhizobia with RE (routinely, 3 h), since no stimulation of adsorption was observed when RE was added just at the start of the adsorption assay (Table 1, line 2), similar to the result obtained when RE was totally omitted (line 1). The stimulation caused by the 3-h preincubation with RE became less noticeable as the period of final incubation with roots (and their concomitant endogenous exudation) during the adsorption assay was extended beyond 1 h, until at 4 h or more the adhesiveness was no different from that in the control (data not shown). This time point is coincident with that of the maximal rate of adsorption in experiments with nonpreincubated rhizobia (Fig. 1, curve I).

Sometimes the presence of RE restricted to the adsorption assay of FS-preincubated rhizobia caused an inhibition of rhizobial adsorption to the root surface, as compared with a control without RE (data not shown). To avoid any incidence of such effects during the final incubation and to highlight the degree of stimulation of rhizobial adsorption ascribable to preincubation with RE, in all the experiments described below (and prior to the adsorption assay) the controls have been supplemented with RE and experimental flasks have been supplemented with FS to obtain equivalent final compositions in the respective assay media.

In this way, over an extended series of 26 independent experiments, the increment value of 1-h adhesiveness (adhesiveness of rhizobia preincubated with RE minus adhesiveness of the respective paired control, FS-preincubated rhizobia, $A_P - A_0$) gave an average of 0.343 (in units of adhesiveness) with a high degree of significance (Student's t test, $\sigma_{n-1} = 0.260$; $n = 26$; $P < 0.002$); the average stimulation (S) was 94%.

The adhesiveness of *R. meliloti* was also increased by preincubation with RE when its specific adsorption to alfalfa roots was assayed in the presence of saturating concentrations (10^7/ml) of heterologous *R. trifolii* A118 cells (data not shown).

Properties of the stimulatory activity present in alfalfa root exudate. The stimulatory activity of alfalfa root exudate has been found in exudates collected after exudation for a few hours or several days. The elimination of endogenous bacterial contaminants of roots remaining after seed sterilization (8) by a newly developed procedure of incubation with streptomycin before exudation (see Materials and Methods) did not visibly influence the exuded activity. Routinely, a treatment of 2-day-old seedlings with streptomycin followed by exudation for 3 days has been used to obtain exudates that are free of bacteria.

When alfalfa root exudate was dialyzed against FS, the stimulatory activity was retained inside the bag, either with 3,500- or 12,000-molecular-weight-cutoff membranes. The dialyzed RE frequently caused a higher stimulation compared with nondialyzed exudate (data not shown), suggesting that some of these substances might interfere with the development of the effect, or with its assay. Accordingly, the experiments that follow have been performed with dialyzed RE.

Heat treatment of RE by incubating at 100°C for 30 min completely abolished the stimulatory activity ($S_{control}$ = 55%; S_{heated} = 4%). This is also true for nondialyzed exudate.

The stimulatory activity of alfalfa RE was also eliminated by treatment with trypsin and subsequent quenching of proteolytic activity by addition of soybean trypsin inhibitor (see Materials and Methods): $S_{control}$ = 107%; $S_{trypsinized}$ = −8% (average results from two independent experiments). Controls in which inhibited trypsin (premixed with trypsin inhibitor) was added before the preincubation of rhizobia or before the adsorption assay, were not affected in the stimulatory effect, or in adsorption to roots, respectively.

These results suggest that the factor(s) in alfalfa root exudate responsible for the stimulation of rhizobial adsorption to roots is macromolecular and proteinlike. They also show that the factor remains active even after dialysis (which would have largely decreased the concentration of low-molecular-weight substances such as *nod*-inducing flavonoids presumably present in the original exudate [31]). The destruction of activity by heat treatment or by tryptic attack points to the absence of stimulatory activity of any heat- or trypsin-resistant molecules by themselves, either remaining in RE after dialysis or present in the original exudate.

Effect of NO_3^- nutrition on the stimulatory activity of alfalfa RE. It is well known that combined nitrogen inhibits the complete process of symbiotic association and nitrogen fixation (39, 41). To study the influence of the plant N nutritional status on the level of stimulatory activity in alfalfa RE, we grew the seedlings in FS supplemented with 5 mM KNO_3. The resulting RE(NO_3^-) was processed in the same way as the RE of plants grown in N-free medium (control) and was dialyzed against FS without NO_3^-, as indicated above. No stimulatory activity was detected in this NO_3^--grown RE (average results of two independent experiments, $S_{N\text{-free}}$ = 100%; $S_{N\text{-plus}}$ = −4%), indicating that the activity is then under negative regulatory control by the N nutritional status of the plant.

Elimination of the stimulatory activity present in alfalfa RE by pretreatment with *R. meliloti*. The following experiments were done to explore whether the interaction of RE with rhizobia affected at all the subsequent, continued presence of the stimulatory factor in solution. Alfalfa RE was pretreated for 30 min with 10^7 cells of *R. meliloti* L5-30 per ml from a late-exponential-phase culture, and the rhizobia were then separated by filtration through a sterilizing, nonadsorbent membrane. When the remaining stimulatory activity in the filtrate (designated pretreated RE) was assayed as described above, while the stimulatory activity of the control treated without bacteria was 134%, the activity that remained in *R. meliloti*-treated RE was −12%. The nature of the phenomenon of disappearance of the stimulatory activity from solution by pretreatment with rhizobia is unknown. In the present work the effect is designated as elimination of the RE factor from solution.

To study the elimination effect further, equal amounts of a preparation of RE were pretreated for 30 min with a range of concentrations of *R. meliloti* L5-30 in the late exponential phase of growth. The stimulatory activity remaining in the sterile filtrates (Fig. 2) decreased in direct relation to the

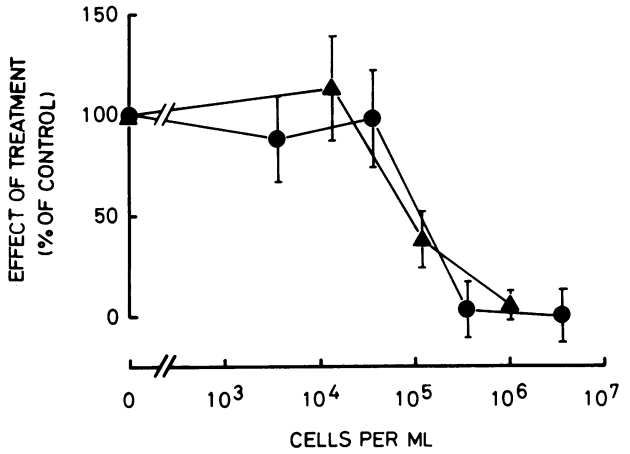

FIG. 2. Elimination of the stimulatory activity present in alfalfa RE by pretreatment with *R. meliloti* and dependence on rhizobial concentration and stage of growth. Alfalfa RE was pretreated with the indicated concentrations of *R. meliloti* L5-30 cells for 40 min at 28°C. After centrifugation and filter sterilization of the supernatant, its stimulatory activity for rhizobial root adsorption was assayed as indicated in Table 1. Symbols: ●, stimulation after pretreatment with late-exponential-phase *R. meliloti* L5-30; ▲, stimulation after pretreatment with stationary-phase *R. meliloti* L5-30. Vertical bars indicate 95% confidence intervals.

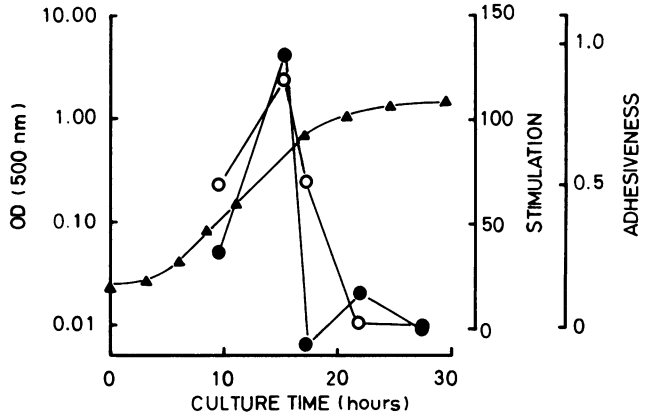

FIG. 3. Effect of culture age on the stimulation of *R. meliloti* for adsorption to roots. The growth curve of *R. meliloti* L5-30 in minimal medium (21) was monitored by measuring optical density readings at 500 nm [OD (500 nm)] (▲). Culture samples collected at the indicated times, serially diluted in FS, and preincubated at approximately 5×10^3 cells per ml with alfalfa RE were assayed for stimulation of adsorption (●) as indicated in Table 1. The same samples diluted in FS were assayed for direct rhizobial adsorption to roots (○) without any preincubation.

concentration of rhizobia, following an apparently near-stoichiometric titration curve in the interval from 3×10^4 to 3×10^5 rhizobia per ml. Similar results were obtained when factor-eliminating bacteria had been isolated and washed in FS prior to pretreatment of RE (results not shown).

These results, although not providing definite indications about the fate of the factor that had disappeared, do indicate that (i) the factor-eliminating activity resides in the rhizobia themselves; (ii) the process of elimination of the stimulatory activity from solution by rhizobia is a relatively fast event, which is completed in less than 30 min; and (iii) a limited number of rhizobia (fewer than 10^6 bacteria per ml) are required for complete elimination of the factor in our RE preparations.

Interaction of *R. meliloti* with dialyzed alfalfa RE as a function of rhizobial growth phase. The stimulation of adsorption of *R. meliloti* L5-30 to alfalfa roots by its preincubation with alfalfa RE was strongly dependent on the growth phase of the rhizobial culture (Fig. 3), being maximal with late-exponential-phase rhizobia and absent with stationary-phase cells. It is noteworthy that the growth phase dependence of this response was parallel to that of the ability of nonpreincubated *R. meliloti* L5-30 to perform adsorption to roots (Fig. 3). The highly transient characteristic of the stimulation response could explain, at least in part, the dispersion of values of stimulation obtained in independent experiments (40 to 200%), in which, despite careful standardization of the experimental procedure, uncontrolled differences in the state of the culture at the time of harvest might have caused considerable variation in the response to the stimulatory factor.

To find whether this dependence on the bacterial growth phase also applies to the ability of rhizobia to eliminate RE from solution, *R. meliloti* L5-30 cells from a stationary-phase culture unable to be stimulated by RE (Fig. 3) were used at various concentrations to pretreat RE as described above. The assay of the remaining stimulatory activity in the respective RE filtrates indicated that these stationary-phase rhizobia were actually able to cause elimination of the stimulatory activity; moreover, they did so with the same efficiency as cells from a late exponential culture (Fig. 2). The ability of *R. meliloti* to perform this elimination is thus constitutive; consequently, elimination of the RE factor is not the event in the stimulatory process which is subject to limitation by the stage of growth.

Expression of symbiotic specificity in the interaction between alfalfa RE and *R. meliloti*. RE from alfalfa and from white clover (which belong to two incompatible legume inoculation groups, respectively) were each tested for their ability to stimulate *R. meliloti* for adsorption to alfalfa roots. The results (Table 2) indicate that although the homologous combination was stimulatory, clover RE, which is active in stimulating its homologous symbiont *R. trifolii* (Table 2, footnote *c*), could not replace alfalfa RE in its stimulatory activity on *R. meliloti*. Therefore, the stimulation of *R. meliloti* for adsorption to roots by prior interaction with the RE factor appears to express the specificity of the *R. meliloti*-alfalfa symbiotic association.

Experiments whose results are given in Table 3 have shown that this specificity is also operating in the short-term

TABLE 2. Stimulation of *R. meliloti* L5-30 for root adsorption by preincubation with homologous or heterologous dialyzed RE

Preincubation medium	Adhesiveness[a]	Stimulation, %[b]
FS	0.20 ± 0.02	
Alfalfa RE	0.42 ± 0.04	+110
FS	0.24 ± 0.02	
White clover RE[c]	0.23 ± 0.02	−4

[a] Values are weighted averages of two independent experiments. Results are given with 95% confidence intervals.
[b] See Table 1, footnote *d*.
[c] In a control experiment, white clover RE was active in stimulating *R. trifolii* adsorption to clover (*S* = 200%).

TABLE 3. Stimulatory activity remaining in dialyzed RE of alfalfa after pretreatment with different species of rhizobia

Strain used to pretreat RE[a]	Adhesiveness of *R. meliloti* L5-30 preincubated with:		
	Pretreated RE	FS	Stimulation, %[b]
Expt A			
None	1.00 ± 0.15	0.40 ± 0.06	+150
R. meliloti L5-30	0.33 ± 0.05	0.40 ± 0.06	−17
R. trifolii A118	1.36 ± 0.20	0.58 ± 0.08	+134
Expt B			
None	0.58 ± 0.07	0.30 ± 0.04	+93
R. meliloti L5-30	0.42 ± 0.05	0.42 ± 0.05	0
R. meliloti 2011	0.34 ± 0.06	0.45 ± 0.08	−24
Expt C			
None	0.26 ± 0.04	0.16 ± 0.03	+62
R. trifolii A118	0.35 ± 0.05	0.17 ± 0.03	+106
R. leguminosarum 248	0.49 ± 0.06	0.26 ± 0.04	+88
R. phaseoli CE-3	0.24 ± 0.03	0.07 ± 0.01	+243

[a] Late exponential cultures of the indicated strains were centrifuged at 10,000 × *g* for 15 min; pellets were resuspended in alfalfa RE and diluted to approximately 10^6 to 10^7 bacteria per ml. After incubation at 28°C for 30 min, bacteria were separated by centrifugation and the supernatant was filtered through a 0.2-μm polycarbonate membrane. The sterile filtrate was used to preincubate fresh *R. meliloti* L5-30. Preincubated rhizobia were finally assayed for adsorption to alfalfa roots.

[b] See Table 1, footnote *d*.

phenomenon of elimination of the stimulatory activity from alfalfa RE by rhizobia. In each of three independent experiments, A, B, and C, alfalfa RE was pretreated for 40 min with about 10^7 bacteria of either homologous *R. meliloti* (experiments A and B) or heterologous rhizobia (experiments A and C) per ml; after filtering out the bacteria, the stimulatory activity remaining in the pretreated RE was assayed by performing a 3-h preincubation of fresh *R. meliloti* and a subsequent 1-h adsorption assay on alfalfa roots. Pretreatment of RE with homologous strains *R. meliloti* L5-30 (experiments A and B) or 2011 (experiment B) caused, in both cases, total elimination of the stimulatory activity, as mentioned above. Instead, when the pretreating strain was heterologous—*R. trifolii* A118 in experiment A and either *R. trifolii* A118, *R. leguminosarum* 248, or *R. phaseoli* CE-3 in experiment C—it failed to eliminate the stimulatory activity, which remained in the pretreated filtrate at levels apparently even higher than in the untreated control. The lack of elimination was observed with substantial concentrations of heterologous rhizobia, one or more orders of magnitude higher than the levels of homologous rhizobia which caused total elimination (Fig. 2). This indicates a high degree of selectivity and discriminatory power against heterologous rhizobia in the interaction for elimination between the alfalfa RE factor and the microsymbiont, even prior to the development of the stimulatory effect.

DISCUSSION

Previous studies in this laboratory (9) have demonstrated that in the process of adsorption of free-living *R. meliloti* (in low concentration) to alfalfa roots during preinfection, the characteristic specificity of this symbiotic association is already expressed. This fact implies the existence of a very early interaction event(s) between the potential associates in which this specificity already operates. The complexity of the process of adsorption has been further indicated by the finding of an initial lag period of several hours before active adsorption takes place, which suggests that some sort of conditioning precedes its onset. Furthermore, it appeared that this process requires the participation of the root exudate, presumably by interacting with the rhizobia.

The root exudate might conceivably favor the adsorption of rhizobia in at least two nonexclusive ways. (i) The first is through its content of rhizobial attractants (both nonspecific and specific) which, depending on the in situ generation of exudate concentration gradients radially from the root surface, direct rhizobial chemotaxis to roots; this facilitates the rapid adsorption of rhizobia to root surfaces and precocious root infection and nodulation, as shown before (10). (ii) The second is by means of some nondirectional, gradient-independent interaction with the rhizobia, which would cause these to perform any subsequent steps toward adsorption more actively or efficiently.

In the present study, which has dealt exclusively with the second type of phenomenon, rhizobia in homogeneous reaction mixtures in the absence of roots were presented with gradient-free exudate preparations and subsequently assayed for any changes in their root adsorption ability. In this way we have been able to demonstrate that the alfalfa root exudate does have a role in enhancing the ability of *R. meliloti* to perform adsorption to roots (including specific adsorption) and that this modification of rhizobial behavior results from an early interaction(s) of the free-living rhizobia with the root exudate in the rhizosphere, prior to attachment to root surfaces.

This interaction has two salient features, namely its symbiotic specificity and the participation of an alfalfa exudate factor. A preliminary characterization by means of dialysis, heat treatment, and tryptic digestion has suggested that the factor involves at least one macromolecular, proteinlike component of unknown complexity. Exudate flavonoids (with key roles in other symbiotic events as regulators of *nod* gene expression [30, 32] and specific root chemoattractants [7] cannot presently be totally discarded as eventual participants, but this does not appear to be very likely, since dialysis of the exudate, which would have largely decreased their original concentration, did not diminish but, on the contrary, frequently increased the stimulatory activity. Moreover, low-molecular-weight, heat-stable substances in the exudate were unable, by themselves alone, to cause the stimulation for adsorption, and their eventual activity (if any) would require the presence of the heat-labile factor.

Within this time-requiring overall process of stimulation, a rapid interaction between rhizobia and the homologous factor could be detected by the disappearance of the latter from solution. This effect was designated as elimination of the factor, whereby after the rhizobia were filtered out, the filtrate was no longer able to stimulate a new round of freshly added rhizobia. The process of elimination and the stimulation of adsorption share the important characteristic of symbiotic specificity. However, the phenomena have shown differences in some aspects. Thus, elimination was completed in a shorter time and worked with stationery-phase rhizobia as well as with late-exponential-phase cells. Instead, stimulation of adsorption took several hours and required the rhizobia to be in a favorable physiological state, harvested from cultures in a narrow time window around the late exponential phase of growth.

The mechanism of the elimination phenomenon, still unknown, might consist of a sequestration of the free factor (or one of its eventual components) through its specific binding to constitutively expressed receptors on the surface of

homologous rhizobia. These would form complexes stable enough to withstand removal from the suspension by simple filtration of the cells. In longer incubations, these complexes might in turn be the ones to evolve, perhaps through their interaction with some other exudate factor component(s), into modified rhizobia with an enhanced ability to adsorb to roots. As noted above, completion of the stimulatory process at this stage would require the participation of additional specialized components and functions of the *R. meliloti* cells, available only in the late exponential phase of growth.

The dose-response curves of elimination by increasing numbers of rhizobia suggest that each cell (irrespective of its growth phase) has a certain limited capacity for specific reaction with the factor (perhaps as a result of a limited number of binding sites), although with high affinity and discriminatory power. Conversely, a given amount of exudate would be enough to react with, and stimulate, only a limited number of rhizobia.

The nature of the rhizobial modifications caused by the exudate factor(s) is not known. The ability to adsorb to roots might conceivably be enhanced by any of several mechanisms, such as an increased ability for motility or chemotaxis to roots or changes in the chemical nature and reactivity of some surface components of rhizobia, enhancing their affinity for root surfaces. These modifications might involve more than one exudate component, as for the clover exudate lectin and glycosidases (14, 15); they might consist of just a simple binding of the factor(s) to specific sites on the bacterial surface or further changes such as enzymatic attack by the factor to certain surface components or the factor-induced expression of new bacterial components or functions required for binding to roots.

Several coincidences between the characteristics of the stimulation listed here and those of specific adsorption (9) and of the overall infection and nodulation, such as symbiotic specificity, suppression by competitor homologous rhizobia, or a similar dependence on the growth state of the rhizobia and on the N-deprived nutritional status of the plant (6, 6a, 19, 39), are compatible with the notion that the first two phenomena are symbiotic precursors of infection and nodule formation, as formerly proposed for specific adsorption in particular (19). Further support for the involvement of the interaction between the exudate factor and *R. meliloti* in the pathway to nodulation has been provided by the finding that the same exudate-preincubated *R. meliloti* cells that are stimulated for adsorption are able to elicit more precocious nodulation in alfalfa roots (43).

Therefore, the specific interaction between *R. meliloti* and the alfalfa root exudate factor appears to be an initial recognition event in the process of the symbiotic association, occurring in the rhizosphere and not requiring physical contact with the root: soil rhizobia without previous exposure to the host receive a macromolecular signal spontaneously released by the root and start a change of behavior, leading to their specific adsorption to root surfaces.

Parallel to the specific interaction of rhizobia and the exudate factor which stimulates the subsequent symbiotic step of rhizobial adsorption to roots, there coexists in the same rhizosphere another, better known recognition event, namely the specific interaction of rhizobia with the particular set of flavonoid signals exuded by the homologous root (30, 32), in which selectivity is conferred by *nodD* genes (22, 27, 37). Besides directing the chemotactic approach of homologous rhizobia to roots (7) (which favors root adsorption [10]), such interaction causes another modification of the bacteria, this time by inducing their *nod* genes, the host range *nodH* gene among others (18), to produce alfalfa-specific nodulation signals such as the NodRm1 tetrasaccharide (28) and promoting in turn other subsequent symbiotic steps, such as the deformation of root hairs and the induction of cortex cell division and nodule initiation (29). We do not know which genes of *R. meliloti* are involved in the specific interaction with the exudate factor or in the process of stimulation of adsorption. In particular, it is not known whether *nodD* and host range *nodH*, *nodFEG*, and *nodPQ* genes participate in these phenomena.

The existence of those two types of initial rhizobium-exudate interaction is remarkable and indicates that at least at this stage, the pathway of the association is not necessarily linear, but may run through parallel, simultaneous courses converging at some step. Concurrently, the strong discrimination by the factor against interaction with heterologous rhizobia would preclude any stimulation for their adsorption to roots and prevent their competition in the occupation of target sites. This is consistent with the usual observation that the process of root infection and nodulation is not interfered with by the presence of heterologous rhizobia even in large concentrations.

ACKNOWLEDGMENTS

We thank Alberto N. Sarachu and O. Mario Aguilar for critical discussions and Hans Ljunggren for advice, encouragement, and hospitality.

L.G.W. has been awarded fellowships from CONICET. G.F. is a member of the Research Career of CONICET. This work was supported by grants from SECYT, CONICET, and CICBA (all from Argentina) and SAREC Cooperation Project (from Sweden).

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. **Bauer, W. D.** 1981. Infection of legumes by rhizobia. Annu. Rev. Plant Physiol. **32**:407–449.
3. **Bergman, K., M. Gulash-Hoffee, R. E. Hovestadt, R. C. Larosiliere, P. G. Ronco II, and L. Su.** 1988. Physiology of behavioral mutants in *Rhizobium meliloti*: evidence for a dual chemotaxis pathway. J. Bacteriol. **170**:3249–3254.
4. **Bhagwat, A. A., and J. Thomas.** 1982. Legume-*Rhizobium* interactions: cowpea root exudate elicits faster nodulation response by *Rhizobium* spp. Appl. Environ. Microbiol. **43**:800–805.
5. **Bhagwat, A. A., and J. Thomas.** 1983. Legume-*Rhizobium* interactions: role of cowpea root exudate in polysaccharide synthesis and infectivity of *Rhizobium* spp. Arch. Microbiol. **136**:102–105.
6. **Bhuvaneswari, T. V., K. K. Mills, D. K. Crist, W. R. Evans, and W. D. Bauer.** 1983. Effects of culture age on symbiotic infectivity of *Rhizobium japonicum*. J. Bacteriol. **153**:443–451.

6a. **Caetano-Anollés, G.** 1986. Ph.D. thesis. Universidad Nacional de La Plata, La Plata, Argentina.

7. **Caetano-Anollés, G., D. K. Crist-Estes, and W. D. Bauer.** 1988. Chemotaxis of *Rhizobium meliloti* to the plant flavone luteolin requires functional nodulation genes. J. Bacteriol. **170**:3164–3169.
8. **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.
9. **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.
10. **Caetano-Anollés, G., L. G. Wall, A. T. De Micheli, E. M. Macchi, W. D. Bauer, and G. Favelukes.** 1988. Role of motility and chemotaxis in efficiency of nodulation by *Rhizobium meli-*

loti. Plant Physiol. **86**:1228–1235.
11. **Currier, W. W., and G. A. Strobel.** 1977. Chemotaxis of *Rhizobium* spp. to a glycoprotein produced by birdsfoot trefoil roots. Science **196**:434–436.
12. **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.
13. **Dazzo, F. B., and G. L. Truchet.** 1983. Interactions of lectins and their saccharide receptors in the *Rhizobium*-legume symbiosis. J. Membr. Biol. **73**:1–16.
14. **Dazzo, F. B., G. L. Truchet, J. E. Sherwood, E. M. 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.
15. **Dazzo, F. B., G. L. Truchet, J. E. Sherwood, E. M. Hrabak, and A. E. Gardiol.** 1982. Alteration of the trifoliin A-binding capsule of *Rhizobium trifolii* 0403 by enzymes released from clover roots. Appl. Environ. Microbiol. **44**:478–490.
16. **Diaz, 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.
17. **Fåhraeus, G.** 1957. The infection of clover root hair by nodule bacteria studied by a simple glass slide technique. J. Gen. Microbiol. **16**:374–381.
18. **Faucher, C., F. Maillet, J. Vasse, C. Rosenberg, A. A. N. van Brussel, G. Truchet, and J. Dénarié.** 1988. *Rhizobium meliloti* host range *nodH* gene determines production of an alfalfa-specific extracellular signal. J. Bacteriol. **170**:5489–5499.
19. **Favelukes, G., and G. Caetano-Anollés.** 1985. *Rhizobium meliloti* specifically attached to alfalfa roots, obligatory precursor towards nodulation, p. 259. *In* H. J. Evans, P. J. Bottomley, and W. E. Newton (ed.), Nitrogen fixation research progress. Martinus Nijhoff, The Hague, The Netherlands.
20. **Gaworzewska, E. T., and M. J. Carlile.** 1982. Positive chemotaxis of *Rhizobium leguminosarum* and other bacteria towards root exudates from legumes and other plants. J. Gen. Microbiol. **128**:1179–1188.
21. **Götz, R., N. Limmer, K. Ober, and R. Schmitt.** 1982. Motility and chemotaxis in two strains of *Rhizobium* with complex flagella. J. Gen. Microbiol. **128**:789–798.
22. **Györgypal, Z., N. Iyer, and A. Kondorosi.** 1988. Three regulatory *nodD* alleles of diverged flavonoid-specificity are involved in host-dependent nodulation by *Rhizobium meliloti*. Mol. Gen. Genet. **212**:85–92.
23. **Halverson, L. J., and G. Stacey.** 1984. Host recognition in the *Rhizobium*-soybean symbiosis. Detection of a protein factor in soybean root exudate which is involved in the nodulation process. Plant Physiol. **74**:84–89.
24. **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.
25. **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.
26. **Halverson, L. J., and G. Stacey.** 1986. Signal exchange in plant-microbe interactions. Microbiol. Rev. **50**:193–225.
27. **Honma, M. A., M. Asomaning, and F. M. Ausubel.** 1990. *Rhizobium meliloti nodD* genes mediate host-specific activation of *nodABC*. J. Bacteriol. **172**:901–911.
27a. **Lagares, A.** 1989. Ph.D. thesis. Universidad Nacional de La Plata, La Plata, Argentina.
28. **Lerouge, P., P. Roche, C. Faucher, F. Maillet, G. Truchet, J. C. Promé, and J. Dénarié.** 1990. Symbiotic host-specificity of *Rhizobium meliloti* is determined by a sulphated and acylated glucosamine oligosaccharide signal. Nature (London) **344**:781–784.
29. **Long, S. R.** 1989. Rhizobium-legume nodulation: life together in the underground. Cell **56**:203–214.
30. **Maxwell, C. A., U. A. Hartwig, C. M. Joseph, and D. A. Phillips.** 1989. A chalcone and two related flavonoids released from alfalfa roots induce *nod* genes of *Rhizobium meliloti*. Plant Physiol. **91**:842–847.
31. **Peters, N. K., J. W. Frost, and S. R. Long.** 1986. A plant flavone, luteolin, induces expression of *Rhizobium meliloti* nodulation genes. Science **233**:977–980.
32. **Peters, N. K., and S. R. Long.** 1988. Alfalfa root exudates and compounds which promote or inhibit induction of *Rhizobium meliloti* nodulation genes. Plant Physiol. **88**:396–400.
33. **Rolfe, B. G., and P. M. Gresshoff.** 1988. Genetic analysis of legume nodule initiation. Annu. Rev. Plant Physiol. Plant Mol. Biol. **39**:297–319.
34. **Rovira, A. D.** 1969. Plant root exudates. Bot. Rev. **35**:35–57.
35. **Smit, G., J. W. Kijne, and B. J. J. Lugtenberg.** 1989. Involvement of both cellulose fibrils and Ca^{2+}-dependent adhesin in the attachment of *Rhizobium leguminosarum* to pea root hair tips. J. Bacteriol. **169**:4294–4301.
36. **Solheim, B.** 1983. Possible role of lectins in binding rhizobia to host roots, p. 539–547. *In* T. C. Bog Hansen and G. A. Spengler (ed.), Lectins, vol. 3. Walter de Gruyter, Berlin.
37. **Spaink, H. P., C. A. Wijffelman, E. Pees, R. J. H. Okker, and B. J. J. Lugtenberg.** 1987. *Rhizobium* nodulation gene *nodD* as a determinant of host specificity. Nature (London) **328**:337–339.
38. **Stacey, G., A. S. Paau, and W. J. Brill.** 1980. Host recognition in the *Rhizobium*-soybean symbiosis. Plant Physiol. **66**:609–614.
39. **Streeter, J. G.** 1988. Inhibition of legume nodule formation and nitrogen fixation by nitrate. Crit. Rev. Plant Sci. **7**:1–23.
40. **Vincent, J. M.** 1970. A manual for the practical study of the root-nodule bacteria. IBP handbook no. 15. Blackwell Scientific Publications, Oxford.
41. **Vincent, J. M.** 1980. Factors controlling the legume-*Rhizobium* symbiosis, p. 103–129. *In* W. E. Newton and W. H. Orme-Johnson (ed.), Nitrogen fixation, vol. 2. University Park Press, Baltimore.
42. **Wall, L. G.** Unpublished results.
43. **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 and Hall, New York.