APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Aug. 1986, p. 377–382
0099-2240/86/080377-06$02.00/0

Vol. 52, No. 2

Host-Symbiont Specificity Expressed during Early Adsorption of *Rhizobium meliloti* to the Root Surface of Alfalfa†

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Received 11 July 1985/Accepted 26 February 1986

Early (4 h) adsorption of *Rhizobium meliloti* L5-30 in low numbers to alfalfa roots in mineral solution was examined for competition with other bacterial strains. All tested competitor strains decreased the adsorption of L5-30 by extents which depended on the strain and its concentration. The decrease of adsorption by *R. meliloti* competitors (all of them infective in alfalfa) was nearly complete at saturation (97 to 99% decrease). All other heterologous rhizobia and *Agrobacterium tumefaciens* at saturating concentrations (10^6 to 10^7 per ml) decreased adsorption of L5-30 only partially, less than 60%. The differential effects of homologous and heterologous competitors indicate that initial adsorption of *R. meliloti* to the root surface of its host occurs in symbiont-specific as well as nonspecific modes and suggest the existence of binding sites on roots which are highly selective for the specific microsymbiont in the presence of other heterologous bacteria even in very unfavorable (less than 10^{-4}) symbiont-competitor concentration ratios.

The symbiotic association of rhizobia and leguminous roots to form nitrogen-fixing nodules is a symbiont-selective process in which only certain combinations of host and rhizobial species work. Thus, legumes of the genera *Medicago*, *Melilotus*, and *Trigonella* develop symbiotic nodules only in homologous association with *Rhizobium meliloti*, and strains of this species are in turn unable to nodulate other legumes. The restrictive character of the host-*Rhizobium* symbiosis is evidenced throughout the orderly course of association, not only during nodule development and function, but also in the preceding stages, at or before infection thread initiation (15, 17). In the search for the earliest interaction between symbionts at which such specificity might operate, adsorption of free-living rhizobia to root surfaces, particularly root hairs, has been found to be symbiont-selective in several legume systems. This was first demonstrated by Dazzo et al. (6) in a quantitative study of adsorption in the clover-*R. trifolii* association, where the bacterial numbers of hair-bound homologous strains largely exceeded those of heterologous *R. meliloti*. Selective adsorption would be the early recognition step at which, according to Dazzo and Hubbell (5), a root lectin would specifically cross-bridge surface components in both symbionts. Qualitative evidence of specific adsorption to root hairs has also been obtained in the associations *Glycine soja-R. japonicum* (21) and *Pisum sativum-R. leguminosarum* (13). However, other studies, in which overall binding of rhizobia to the whole root surface (instead of root hairs) was measured, failed to reveal selective mechanisms in the adsorption process; this has been the case in pea (2, 4), clover (19), and cowpea and soybean (18). In agreement with the latter observations, we have reported adsorption of homologous as well as heterologous bacteria to alfalfa and clover roots, suggesting that overall adsorption does not reflect the specificity of the symbiotic association (3). Similar results at higher bacterial concentrations have been obtained by Lafrenière et al. (14). All these results may arise from the existence of nonspecific as well as specific modes of bacterial binding to roots (7); the latter would be obscured when the former prevails. By introducing a differential assay for rhizobia loosely or firmly bound over the whole root surface, Jansen van Rensburg and Strijdom (12) have been able to show that, in the alfalfa-*R. meliloti* and clover-*R. trifolii* associations, firm binding (after prolonged contact between symbionts) was largely host specific.

In this paper we explore the possible existence of symbiont-specific and nonspecific modes of rhizobial adsorption to alfalfa roots. This question is approached in a quantitative study of the inhibitory effects on early adsorption of *R. meliloti* caused by homologous and heterologous bacteria.

(A preliminary account was presented to the 3rd International Symposium on Microbial Ecology, East Lansing, Mich., 7–12 August 1983 [abstract E-11, p. 39].)

MATERIALS AND METHODS

Bacterial cultures and plant material. The strains used in this study are listed in Table 1. *R. meliloti* L5-30 (resistant to streptomycin) was obtained from G. Martínez-Drets, Montevideo, Uruguay. *R. meliloti* L5-30-1, a rifampin-resistant derivative of L5-30 which retains the parental ability for adsorption and nodulation, was obtained by mutagenesis with diethylsulfate (A. T. De Micheli, this laboratory). *R. meliloti* 1021 (resistant to streptomycin) was a gift of F. M. Ausubel, Cambridge, Mass. *Rhizobium* sp. strain G1 was isolated from spontaneous nodules of *Acacia floribunda* (provided by G. Pozzo Ardizzi-Fidel). Cultures were maintained on YEM agar and grown to late exponential phase (OD_{500}, 0.3 to 0.4) in YEM liquid medium as previously described (3). Seeds of alfalfa (var. Dawson) were surface-sterilized and germinated on water-agar plates (3).

Studies of bacterial adsorption to roots in the presence of competitor bacteria. Root adsorption of the standard *R. meliloti* indicator (Idr) antibiotic-resistant strain (L5-30 or its derivative, L5-30-1) was quantitatively assayed as described previously (3) in the absence or presence of competitor bacteria sensitive to the same antibiotic. The culture of the Idr strain (in a final concentration of 2×10^3 bacteria per ml)

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† Dedicated to Luis F. Leloir on the occasion of his 80th birthday, 6 September 1986.

TABLE 1. Adsorption of *R. meliloti* L5-30 strain to alfalfa roots: inhibition by homologous and heterologous competitor bacteria

Competitor strain	Source[a]	Infectivity in alfalfa	log B[b]	% A of Idr L5-30[c]		% Inhibition[d]
				With competitor (A_c)	Without competitor (A_o)	
Homologous						
R. melitloti						
AP3	1	+	6.63	0.05 ± 0.02	3.18 ± 0.59	98.4
AN3	1	+	6.52	0.05 ± 0.02	3.18 ± 0.59	98.4
AN9	1	+	6.55	0.03 ± 0.02	3.18 ± 0.59	98.9
Rh26	1	+	6.58	0.02 ± 0.01	3.18 ± 0.59	99.5
Rm4	2	+	6.61	0.07 ± 0.02	3.18 ± 0.59	97.9
Rm4c	2	+	6.64	0.04 ± 0.02	3.18 ± 0.59	98.6
R41	3	+	6.58	0.07 ± 0.02	3.18 ± 0.59	97.7
N77	3	+	6.54	0.08 ± 0.03	3.18 ± 0.59	97.5
DP-4	4	+	6.47	0.07 ± 0.02	3.18 ± 0.59	97.8
B251	5	+	6.48	0.09 ± 0.03	3.18 ± 0.59	97.0
B19	5	+	6.66	0.04 ± 0.02	3.18 ± 0.59	98.8
U45	6	+	6.93	0.03 ± 0.02	3.06 ± 0.38	98.9
Heterologous						
R. trifolii						
A118	5	−	6.62	1.29 ± 0.22	3.14 ± 0.46	58.9
A4-1a	4	−	6.55	1.69 ± 0.28	3.14 ± 0.46	46.3
A1	4	−	6.60	1.44 ± 0.24	3.14 ± 0.46	54.3
A131	6	−	6.62	1.26 ± 0.15	3.04 ± 0.30	58.6
R. phaseoli						
F45	5	−	6.56	2.41 ± 0.33	2.98 ± 0.40	19.0
F48	5	−	6.62	2.39 ± 0.33	2.98 ± 0.40	20.0
R. leguminosarum						
D94	6	−	6.73	1.95 ± 0.28	2.98 ± 0.40	34.7
D138	5	−	6.69	1.65 ± 0.24	2.98 ± 0.40	44.6
R. japonicum						
USDA6	7	−	6.54	2.61 ± 0.26	3.03 ± 0.30	13.9
USDA24	7	−	6.61	2.13 ± 0.22	3.03 ± 0.30	29.5
USDA110	7	−	6.53	1.81 ± 0.29	3.15 ± 0.46	42.4
USDA117	7	−	6.42	2.53 ± 0.26	3.03 ± 0.30	16.3
USDA122	7	−	6.49	1.87 ± 0.22	3.15 ± 0.35	40.9
USDA136	7	−	6.40	2.28 ± 0.26	3.15 ± 0.35	27.8
USDA138	7	−	6.51	1.82 ± 0.19	3.03 ± 0.30	40.1
USDA143	7	−	6.79	2.34 ± 0.24	3.03 ± 0.30	22.6
Rhizobium spp.						
G1 (acacia)	8	−	6.56	2.83 ± 0.29	3.04 ± 0.30	7.0
32H1 (cowpea)	9	−	6.80	1.96 ± 0.17	2.96 ± 0.33	33.8
A. tumefaciens LBA288	10	−	6.67	1.96 ± 0.31	3.15 ± 0.46	37.6

[a] Sources: 1, S. M. Lesley, Ottawa, Ontario, Canada; 2, J. Olivares, Granada, Spain; 3, A. Kondorosi, Szeged, Hungary; 4, Facultad de Agronomía y Veterinaria, Buenos Aires, Argentina; 5, R. Diéguez, INTA, Castelar, Argentina; 6, M. Barate de Bertalmío, Montevideo, Uruguay; 7, R. Griffin, Beltsville, Md.; 8, this laboratory; 9, J. C. Burton, Milwaukee, Wis.; 10, P. J. J. Hooykaas, Leiden, The Netherlands.

[b] B, Concentration of the competitor (bacteria per ml).

[c] *A* values are given with 95% confidence intervals.

[d] Percent inhibition = $(A_o - A_c)/A_o \times 100$.

diluted with nitrogen-free Fåhraeus solution (10) was mixed with appropriate dilutions of a competitor culture in the same solution. Where indicated, competitor culture filtrates (0.45-μm-pore-size membranes; Millipore Corp.) or filtered bacteria (gently suspended in Fåhraeus solution) were used as replacement for competitor cultures. Incubations of alfalfa seedlings with the bacterial suspensions, subsequent washings, and the selective detection and counting of root-adsorbed Idr rhizobia as microcolonies in contact with the root surface were performed as described previously (3). The Idr-selective antibiotic incorporated into the root-embedding YEM-cycloheximide agar medium was streptomycin (100 μg/ml) for L5-30 or rifampin (40 μg/ml) for L5-30-1. In all cases, controls with seedlings incubated with competitors but without the indicator strain did not develop any microcolonies. The degree of root adsorption of the Idr strain is expressed as adhesiveness, *A* (defined in reference 3).

Growth interference assays. Interference by competitors with growth of the indicator strain on solid media was tested by the method of De Antoni et al. (9). Growth interference in solution was tested in the adsorption assay (3), in which seedlings were omitted. Idr bacteria (10^3/ml) were incubated in Fåhraeus solution for 4 h at 28°C in the absence or presence of a competitor (10^6 to 10^7/ml). Survival and growth of the Idr was followed by sampling at different times for its selective counting in antibiotic plates. Generation times were then calculated and compared.

RESULTS

The rationale of the present experiments is that the adsorption of low numbers of a standard *R. meliloti* strain

(the Idr strain, antibiotic resistant) to alfalfa root surfaces in the presence of different competitor bacteria (antibiotic sensitive) can be selectively quantitated by colony development in an antibiotic-supplemented medium. This has been done by means of the quantitative adsorption assay described in the accompanying paper (3).

Preliminary experiments indicated that when *R. meliloti* U45 (10^7/ml) was used as a competitor, the degree of root adsorption (*A*) of the Idr strain L5-30 (2×10^3/ml) decreased by 99%. In a more detailed study, the concentration of the *R. meliloti* competitor strain was varied between 10^2 and 10^7/ml. Figure 1 shows the results obtained in this way with three competitor-indicator pairs of strains: L5-30 competing against its isogenic derivative Idr strain L5-30-1, and U45 and AP3 competing against nonisogenic Idr strain L5-30. In all three cases root adsorption of Idr was progressively inhibited by increasing concentrations of the competitor, starting at 10^4/ml; inhibition was practically total in the presence of about 10^6 competitor organisms per ml. In a similar experiment in which the heterologous strain *R. trifolii* A118 (noninfective for alfalfa) was used as a competitor, root adsorption of Idr L5-30 was again inhibited by competitor concentrations greater than 10^3/ml. In this case, there was an initial steep effect reaching 35% inhibition at 2×10^4 competitors per ml, but it was followed by a shallower, gradual increase not exceeding 60% inhibition at 10^7/ml (Fig. 1). Differences between *R. meliloti* U45 and *R. trifolii* A118 as competitors in their inhibitory capacities at high concentration did not appear to depend on the particular indicator strain used, since in other experiments each of them was able to inhibit root adsorption of Idr *R. meliloti* 1021 (99.2 and 51.1%, respectively) to the same extent as Idr *R. meliloti* L5-30.

In the preceding experiments competitor bacteria were added to the incubation mixture as portions of cultures also containing nutrients and soluble products of bacterial origin,

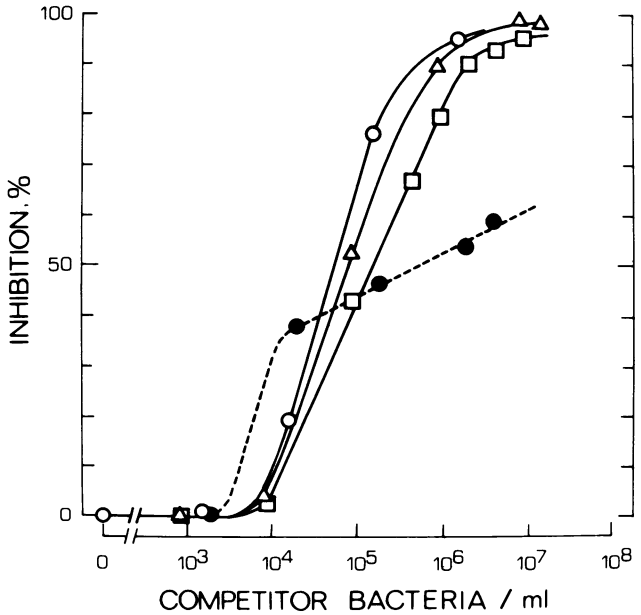

FIG. 1. Inhibition of root adsorption of *R. meliloti* Idr strains by different competitor strains. Symbols: ○, Idr *R. meliloti* L5-30-1, competitor *R. meliloti* L5-30; △, Idr *R. meliloti* L5-30, competitor *R. meliloti* U45; □, Idr *R. meliloti* L5-30, competitor *R. meliloti* AP3; ●, Idr *R. meliloti* L5-30, competitor *R. trifolii* A118.

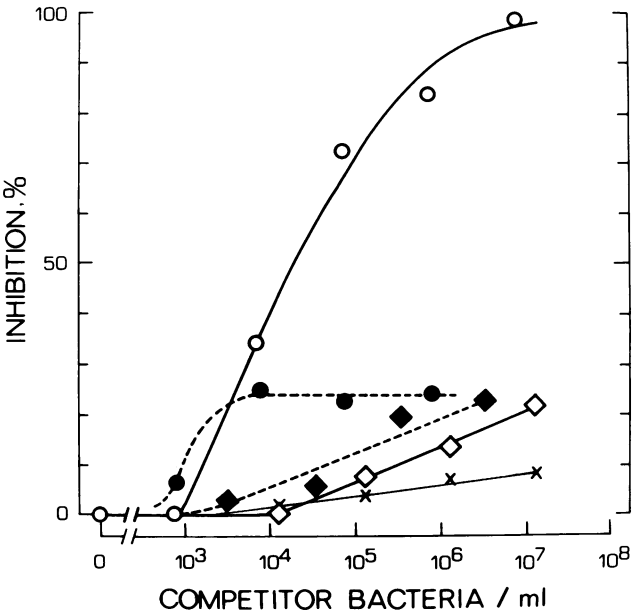

FIG. 2. Inhibition of root adsorption of Idr *R. meliloti* L5-30 by filtered competitor bacteria and by culture filtrates. Symbols: ○, filtered *R. meliloti* U45 cells; ●, filtered *R. trifolii* A118 cells; ◇, *R. meliloti* U45 culture filtrate; ◆, *R. trifolii* A118 culture filtrate; ×, fresh YEM medium. Filtrates are represented as equivalent numbers of bacteria contained before filtration; 1 ml of fresh YEM medium was taken to be equivalent to 1.42 (± 0.05) × 10^8 bacteria.

which may have contributed to the observed inhibitory effects. To discriminate between the effects of the bacterial cells and those of the accompanying soluble components, filtered bacteria were compared with their culture filtrates in equivalent amounts as competitors in the root adsorption assay of Idr *R. meliloti* L5-30. The diverging inhibitory patterns of filtered cells of *R. meliloti* U45 and *R. trifolii* A118 were similar to those of the respective whole cultures in Fig. 1 (apart from an unexplained shift of both inhibition curves towards lower competitor concentrations [Fig. 2]). It can be seen that filtered *R. meliloti* U45 in high concentration (8×10^6/ml) again caused complete (98.7%) inhibition of root adsorption of Idr L5-30. With filtered *R. trifolii* A118, a limited (24%) inhibition was found, which was obtained with low levels of competitor (8×10^3/ml) and could not be surpassed even with a 100 times higher concentration of filtered cells. The respective culture filtrates led to a gradual inhibition of Idr adsorption that was moderate even in high concentrations (21% for an equivalent of 4×10^7 *R. meliloti* U45 per ml and 23% for an equivalent of 4×10^6 *R. trifolii* A118 per ml); fresh medium was only slightly inhibitory. These results suggest that the culture medium may have been responsible for the gradual increase of inhibition with higher doses of *R. trifolii* A118 competitor (Fig. 1).

We conclude that full inhibition by *R. meliloti* U45 of adsorption of Idr L5-30 to alfalfa roots, distinct from the partial, limited inhibition by *R. trifolii* A118, indicate qualitatively different competitive behavior for these strains. These diverging behaviors appear to be characteristic properties of the competitor bacterial cells proper, regardless of and unmasked by the presence of partially inhibitory culture medium components.

These studies were extended to several rhizobial strains representing the various legume cross-inoculation groups

and to agrobacteria. Inhibition of adsorption of the Idr *R. meliloti* L5-30 to alfalfa roots was assayed as described in the presence of competitor bacteria in high numbers (10^6 to 10^7/ml; approximately 10^4 per Idr bacterium) added as whole cultures. Twelve competitor strains of the homologous *R. meliloti* group—all of them able to nodulate alfalfa—caused complete (97 to 99.5%) inhibition of indicator adsorption (Table 1). In contrast, 18 heterologous strains, including *R. trifolii*, *R. leguminosarum*, *R. phaseoli*, *R. japonicum*, and *Rhizobium* spp. (cowpea, acacia), as well as one strain of *A. tumefaciens*, none of which are infective for alfalfa, were only partially inhibitory to root adsorption of Idr L5-30 (ranging from 7.0 to 58.9% inhibition). It is likely that in the experiments with filtered competitors instead of whole cultures (cf. filtered *R. trifolii* A118, above), heterologous but not homologous strains would have been even less inhibitory.

Our results indicate a pattern of competitor behavior in which all homologous *R. meliloti* strains are completely inhibitory and all heterologous strains are only partially inhibitory. Both groups are clearly differentiated (*t* test, $P < 0.001$) and separated by a gap of not less than 40% in their maximal inhibitory abilities.

The inhibitory effects by the various homologous and heterologous competitors were not caused by any interference or metabolic effect in the presence of the competitor directed against the indicator strain. For competitor *R. meliloti* strains this could be shown by the results in tests of interference for growth in solid media and in Fåhraeus solution. In solid media only 3 (AN3, Rh26, and DP-4) of 12 strains tested showed any interference against L5-30; in Fåhraeus solution, the generation time of L5-30 alone (200 ± 16 min) was not significantly altered (*t* test, $P > 0.2$) in the presence of any of the five strains AN3, Rh26, DP-4, U45, or AP3. As for heterologous competitors causing only partial inhibition even at high concentration, the limit plateau of 24% in the inhibition curve of washed *R. trifolii* A118 from 10^4 bacteria per ml upwards (Fig. 2) indicates the absence of any dosage-dependent competitor effects, such as interference, or decrease in the availability of oxygen or other nutrients.

DISCUSSION

Different bacteria in low concentration, including *R. meliloti*, have been shown to adsorb to alfalfa roots in a liquid milieu (3) regardless of their ability to infect and nodulate the host. The results reported here indicate that the degree of early (4 h) adsorption of an infective Idr strain, *R. meliloti* L5-30, to alfalfa roots is decreased when another bacterial strain is present during the incubation. This effect, which requires solely the presence of the competitor cells and is not due to bacterial interference or competition for nutrients, can be considered to result from the competition between both strains for binding to the root surface. Under our experimental conditions in which the indicator strain was present at a very low concentration (2×10^3/ml), the inhibition of its early adsorption has been found to depend on the concentration and nature of the competitor strain. The effect increased gradually with concentration until at about 10^4 to 10^6 or more competitors per ml (depending on the strain), maximal inhibition was reached. The shape of the curves of inhibition versus competitor dose suggest that, at those moderate concentrations, saturation of the inhibitory effect by the competitor was obtained. When different competitor strains were tested at high concentration for their inhibitory effect, they could be grouped with high statistical confidence into two distinct, mutually exclusive classes: strains that inhibit adsorption of the indicator totally (97% or more), comprising all alfalfa-infective, homologous strains of *R. meliloti*, and bacteria that cause partial inhibition (not exceeding 60%), which include all heterologous rhizobia noninfective in alfalfa as well as *A. tumefaciens*. Particularly revealing was the demonstration of a limit to the partial inhibition by the heterologous competitor, *R. trifolii* A118, which could not be overrridden even with competitor-indicator concentration ratios as high as 10^4. These results indicate that (i) during early adsorption of the symbiont to alfalfa roots, total (as opposed to limited) inhibition by competitors is an exclusive attribute of the homologous species *R. meliloti*; (ii) early adsorption of *R. meliloti* to alfalfa roots is heterogeneous (in agreement with other indirect evidence of a statistical nature, reported by us [3]); and (iii) the fraction of *R. meliloti* indicator adsorption which is resistant to heterologous inhibition, but is suppressed only by *R. meliloti* competitor strains, represents symbiont-selective adsorption.

Although the data presented here do not allow a detailed analysis of the kinetics of adsorption and competition, they are consistent with a model of root adsorption in which the indicator as well as the competitor strain would be able to occupy and share certain classes of sites on the root surface. The sites belonging to each class would be present in finite, moderate numbers and possess considerable tendency to be occupied (affinity) by the indicator and the competitor. These assumptions imply site saturation at low to moderate bacterial concentrations and reciprocal competition between strains for adsorption to common target sites. Sites would fall into two categories: specific sites accessible only to homologous rhizobia and nonspecific ones able to bind both homologous and heterologous bacteria. In the absence of competitors, the Idr strain *R. meliloti* L5-30 would adsorb to both specific and nonspecific sites. Competitor bacteria in high numbers, if homologous, would be able to occupy and saturate both kinds of sites, making them unavailable to the Idr. Heterologous competitors would be able to do so only in sites that are nonspecific while leaving specific sites fully accessible to the Idr. According to this model, residual adsorption of the *R. meliloti* Idr strain in the presence of saturating concentrations of a suitable heterologous competitor would reveal the occurrence and availability of specific sites. The preceding assumptions account for (i) the observed completion of inhibition of indicator adsorption by competitors at limited concentrations and (ii) the clear-cut difference in the inhibition patterns of homologous versus heterologous competitor strains. The differences in inhibitory effects by the various heterologous competitors in Table 1—where no attempts were made to optimize the conditions for each competitor strain—might arise from heterogeneity in the binding properties of the nonspecific sites compounded by expected behavioral differences in the nonspecific binding response from widely diverse bacterial strains. An alternative model, in which all homologous and heterologous bacteria would adsorb to common sites but only the homologous rhizobia would attain the condition of specific binding can be ruled out, since, contrary to our results, it predicts that heterologous competitors in high concentration would totally inhibit the adsorption of the homologous Idr strain.

Rhizobia, which in the free-living state populate the highly particulate medium of soil, may be expected to bind to various kinds of surfaces. Hydrophobic surfaces (16) and the

root surface of nonlegumes (20) support rhizobial binding. Nonsymbiotic adsorption of rhizobia to legume roots has been described in several instances (2–4, 6, 13, 14, 18, 19). In some of these studies adsorption was examined from very low bacterial concentrations upwards (3, 18), and in the *R. japonicum*-soybean association at least, saturation was not reached even at high (10^9/ml) levels of inoculation (18). Root-washing procedures in these studies supported the assumption that at least a large proportion of the root-associated bacteria were surface bound. While the overall degree of adsorption differed widely among strains, it failed to show any definite relation to the symbiotic relatedness of the host-*Rhizobium* pair (3, 14, 18), and the lack of saturation at high rhizobial concentrations (in the *R. japonicum*-soybean association) was taken to indicate that homologous adsorption was largely nonspecific and complex (18).

These results with legume roots then seem to be one particular example of the rhizobial property of nonspecific binding to surfaces. However, definite indications of selective adsorption to legume roots were recorded when observations were confined to a particular fraction of bound rhizobia considered to be involved in the symbiotic process (such as rhizobia adsorbed only to root penetration sites, namely root hairs [6], or bound by special forces, e.g., firmly anchored to roots [12]). In our case, rhizobial adsorption in low numbers to roots under competition with large concentrations of heterologous rhizobia is the restrictive condition which allowed selective detection and counting of rhizobia specifically adsorbed to the host. The subject of specific interaction of *R. meliloti* with alfalfa during root adsorption has been addressed recently also by Lafrenière et al. (14): when alfalfa roots were inoculated with *R. meliloti* singly or in combination with another homologous or heterologous strain (both in equal, high numbers, around 10^8 cells per ml), the proportion of cells of each strain adsorbed (adhesiveness [3]) appeared to be essentially independent of its concentration and of the presence of the other strain. The results were interpreted by the authors as suggesting that *R. meliloti* cells are adsorbed to very specific sites on alfalfa roots—a view that basically agrees with part of our own conclusions. However, their data do not support this suggestion unequivocally, since the evidence indicating absence of saturation by homologous or heterologous strains—a fact which, according to Pueppke (18), implies that adsorption in each case is largely nonselective—and the lack of competition between homologous strains leave open the question of the identity or specificity of occupied sites.

In fact, the quantitative study of specific rhizobial adsorption to roots in the presence of concentrated inocula (as used by Lafrenière et al.) is subject to uncertainties, one of these arising from the early association to roots of bacterial aggregates of considerable size (observed by Dazzo et al. [8] in clover root hairs during phase 1A-specific attachment and by Stacey et al. [21] in soybean root hairs). In these cases, total numbers of bacteria associated to the root (resistant to washing) may include a large proportion of cells physically detached from the root surface, although bound to it through their inclusion in bacterial aggregates, and therefore excluded from direct adsorption mechanisms operating at the bacteria-root interface. Those high numbers of aggregated bacteria would then tend to mask the relatively minor quantities of bacteria truly adsorbed to sites on the root surface, which would be involved in saturation and competition phenomena. Contrary to this, homologous rhizobia attach to root hairs as single cells when light inocula are used (Dazzo et al. [8]). Such has been the case in our experiments, where (as previously discussed [3]) the very dilute indicator rhizobia would have been adsorbed singly and sparsely distributed along the root surface.

Dazzo et al. (8) have recently studied in detail the orderly evolution of attachment in the clover root hair-*R. trifolii* system and described it as a sequence of phases: a very early, specific binding of rhizobia to hairs as clumps or as polarly attached single bacteria (referred to above) easily detached by shear or by specific hapten elution (phases 1A and 1C), which is followed after 12 to 24 h by hapten-irreversible, firm anchoring of rhizobia to the hair surface, mediated by a mesh of microfibrils (phase 2). Similarly, a late phase 2-like firm attachment of *R. trifolii* and *R. meliloti* to the overall root surface of clover and alfalfa, respectively (7 days after inoculation), was found to be largely host-specific (12). In our studies, specific adsorption of *R. meliloti* to alfalfa roots has been shown to occur after contact for 4 h, or even as short as 1 h (L. G. Wall and G. Caetano Anollés, unpublished data). Such short terms and the release of these specifically adsorbed rhizobia from alfalfa roots (unpublished data) by washing procedures designed for the selective detachment of loosely bound rhizobia (12) suggest that our observations refer to very early, phase 1-like, specific rhizobial adsorption.

The experimental approach introduced in this work has allowed us to demonstrate that, in the symbiotic association of *R. meliloti* with alfalfa, a specific interaction of both associates, implying their mutual recognition, occurs, as in other *Rhizobium*-legume symbioses (5, 13, 21), during preinfection at the step of initial adsorption to the root surface. It has also shown the high discriminatory power of the process for specific adsorption, which rejects heterologous bacteria many orders of magnitude more concentrated than symbiotic rhizobia. With the same approach, we have recently demonstrated the role of this selective step as an obligatory precursor of root infection (11). It should be useful also to study the properties and requirements of specific adsorption and its preceding events, the components of both symbionts involved, and the distribution of specifically adsorbed *R. meliloti* cells along the root surface. In this regard, preliminary observations in experiments with the heterologous *R. trifolii* A118 competitor (such as those shown in Fig. 1 and 2) suggest that specifically adsorbed *R. meliloti* L5-30 would be evenly distributed along the roots, without any particular preponderance for the young or the mature zones. Confirmation of this would be interesting in view of the reported relative predominance in alfalfa of fertile sites for nodulation in the younger portions of the root (1).

ACKNOWLEDGMENTS

We thank R. Maronna for advice on statistical matters, F. B. Dazzo, W. D. Bauer, and B. Rolfe for valuable discussions, and scientists mentioned in Materials and Methods and Table 1 for generously providing strains.

This work was supported by grants from SECYT, CONICET, CICBA (all of Argentina) and by the United Nations Development Programme (UNESCO-RLA 78/024). G.C.A. has been the recipient of fellowships from CICBA and CONICET.

ADDENDUM IN PROOF

A recent article (J. Badenoch-Jones, D. J. Flanders, and B. G. Rolfe, Appl. Environ. Microbiol. **49**:1511–1520, 1985), which reached us after the present paper had been submitted, has reported a careful quantitative study of rhizobial

association with clover roots. In that study, direct optical microscopic examination of numbers of rhizobia adsorbed to the legume root hairs did not give any indication of host-symbiont specificity at this step, at variance with previous reports (6). These results point to the limitations of optical microscopic methods for these studies and stress the usefulness of alternative approaches such as the present one.

LITERATURE CITED

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