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Quantitation of Adsorption of Rhizobia in Low Numbers to Small Legume Roots

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Bacteria adsorbed in low numbers to alfalfa or clover root surfaces were counted after incubation of seedlings in mineral solution with very dilute inocula (less than 10^5 bacteria per ml) of an antibiotic-resistant strain under defined conditions. After specified washing, bacteria which remained adsorbed to roots were selectively quantitated by culturing the roots embedded in yeast extract-mannitol-antibiotic agar and counting the microcolonies along the root surface; the range was from about 1 bacterium per root (estimated as the most probable number) to 50 bacteria per cm of root length (by direct counting). This simple procedure can be used with any pair of small-rooted plant and antibiotic-resistant bacterium, requires bacterial concentrations comparable to those frequently found in soils, and yields macroscopic localization and distribution data for adsorbed bacteria over the root surface. The number of adsorbed bacteria was proportional to the size of the inoculum. One of every four *Rhizobium meliloti* cells adsorbed in very low numbers to alfalfa roots resulted in the formation of a nodule. Overall adsorption of various symbiotic and nonsymbiotic bacterial strains to alfalfa and clover roots did not reflect the specificities of these legumes for their respective microsymbionts, *R. meliloti* and *R. trifolii*.

Interactions between soil bacteria and plant roots lead to a variety of associative phenomena, among which symbiotic as well as parasitic or pathogenic relationships are important to plant life and productivity. Many of these associations are characterized by bacterial penetration and invasion of the root tissues. In those cases, an early stage in the developmental pathway is attraction and adsorption of soil bacteria to the root surface. One such instance is found during the preinfection phase in the nitrogen-fixing symbiosis between rhizobia and leguminous roots. In this process, soil rhizobia are initially attracted to the developing roots, colonize the rhizosphere, attach to the root hairs, and cause their deformation and curling. Subsequent rhizobial infection of the root tissues via penetration of the root hairs leads to the formation of a symbiotic nodule (7).

Rhizobial adsorption to legume roots has been of particular interest because in some associations the selectivity of the host-*Rhizobium* pairing characteristic of nodule formation is expressed at this early stage (5, 11, 12, 18, 19, 27). Several approaches have been used to detect and quantitate bacterial adsorption to roots, including optical and scanning electron microscopic observation (1a, 11, 12, 26, 27, 29), counts of radiolabeled bacteria (1a, 4, 6, 18, 19), and plate counts of adsorbed bacteria either after release (17, 23) or in root homogenates (20, 22, 26). Many of these studies have used high numbers of cells in the inoculant suspension and have measured large populations of adsorbed bacteria. In this paper we describe a method for enumeration of root-adsorbed bacteria in very low numbers, obtained with inoculum concentrations reflecting those frequently encountered in soils. The procedure, which is simple and can be used with any small-rooted plants and any bacteria, allows the study of adsorption of rhizobia to alfalfa and clover roots.

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MATERIALS AND METHODS

Microorganisms and plant material. The strains used in this work are listed in Table 1. *Rhizobium meliloti* U45 (Plan Agropecuario, Uruguay) and *R. trifolii* A118 (INTA, Castelar, Argentina; original strain TA1, from CSIRO, Australia) were used to isolate spontaneous derivatives that were resistant to streptomycin and kanamycin and to streptomycin, respectively, and retained the capacity of nodulation and nitrogen fixation in their hosts. Alfalfa (var. Dawson) and white clover (var. Huia) (both supplied by Alexandre & Cia., Buenos Aires) were used for studies of nodulation and bacterial adsorption to roots.

Maintenance and cultivation of bacteria. For *Rhizobium* and *Agrobacterium* strains, YEM (yeast extract-mannitol medium) contained (in grams per liter): mannitol, 10; NaCl, 0.1; $MgSO_4 \cdot 7H_2O$, 0.2; K_2HPO_4, 0.5; and yeast extract (Difco Laboratories), 0.4. For *Escherichia coli*, NB (nutrient broth) contained (in grams per liter): nutrient broth (Difco), 8; NaCl, 5. For *Bacillus subtilis*, CHT (casein hydrolyzate-tryptophan medium) contained (in grams per liter): casein hydrolyzate (Difco), 1.25; tryptophan, 0.05; glucose, 5; $MgCl_2$, 0.123; $CaCl_2$, 0.056; $(NH_4)_2SO_4$, 2; K_2HPO_4, 14; KH_2PO_4, 6; and sodium citrate, 1. Agar (15 g/liter; Difco), congo red (0.25 g/liter), and antibiotics were added when required.

Stock cultures of rhizobia were maintained on YEM agar. *E. coli* and *B. subtilis* strains were maintained on NB and CHT agar, respectively. Starter cultures were grown in YEM, NB, and CHT liquid media, respectively, to the stationary phase of growth. Inoculum cultures for adsorption assays were grown from starter cultures diluted 100-fold in 10 ml of the respective fresh medium and incubated in a rotary water bath shaker at 120 rpm and 30°C to the desired density. *Rhizobium* strains were tested periodically for symbiotic performance.

TABLE 1. Bacterial strains tested

Strain	Characteristics and resistance (μg/ml)[a]	Infective in alfalfa	Source
R. meliloti			
U45sk	Prototrophic; Str^r (50), Kan^r (50)	+	This paper
U210	Prototrophic; Str^r (100), Kan^r (50)	+	G. Martínez-Drets
L5-30	Prototrophic; Str^r (100)	+	G. Martínez-Drets
R. trifolii A118s	Prototrophic; Str^r (50); infective in white clover	−	This paper
E. coli C74	Prototrophic; Str^r (200)	−	H. Lopardo
B. subtilis			
JA-60-A	Nal^r (150); His^- Met^- Leu^- Su^-	−	J. Alonso
Pb1706	Nal^r (300); Tyr^- His^- Trp^- Aro^- Su^-	−	J. Alonso
A. tumefaciens LBA288	Prototrophic; Nal^r (100), Rif^r (100)	−	P. J. J. Hooykaas

[a] Levels of antibiotic resistance are indicated. Abbreviations: Str, streptomycin; Kan, kanamycin; Nal, nalidixic acid; Rif, rifampin.

Assay for adsorption of bacteria to roots. Seeds were surface-sterilized by immersion for 60 min in 95% ethanol and then for 15 min in 0.2% $HgCl_2$ acidified with 0.5% HCl and rinsed four times with sterile water. Five-day-old seedlings were obtained from seeds germinated in darkness at room temperature on inverted sterile water-agar petri dishes.

Bacterial inoculum cultures in late log phase (OD_{500}, 0.4; the actual concentration of viable bacteria was determined in each inoculum culture by plate counts with the overlay procedure) were diluted in 10-fold steps with nitrogen-free Fåhraeus solution (0.9 mM $CaCl_2$, 0.49 mM $MgSO_4$, 0.74 mM KH_2PO_4, 0.84 mM Na_2HPO_4, and 0.015 mM ferric citrate, pH 7.0) (14) to a final colume of 22.5 ml in 250-ml cylindrical flasks (wide-mouthed, capped; 60 mm internal diameter). A noninoculated control flask was included for all experiments. Fifteen seedlings were gently transferred with sterile forceps into each flask and incubated for 4 h at 28°C and 50 rpm in a rotary water bath shaker (motion radius, 12.5 mm). After the fluid was discarded, the seedlings in the flask were washed by gentle suspension in 25 ml of Fåhraeus solution, shaking in the rotary bath at 120 rpm for 1 min, and immediate discard of the wash fluid. Four consecutive washes were performed. The seedlings were gently laid flat on the bottom of a petri dish, carefully embedded with melted (45°C) agar medium, left to cool and solidify, and finally cultured at 30°C for colony growth. The seedling embedding medium in experiments with rhizobia or with *Agrobacterium tumefaciens* was YEM-congo red agar; with *E. coli* or with *B. subtilis*, it was NB soft agar (7.5 g · l^{-1}). In all cases it contained the required antibiotic in the concentration indicated for each strain in Table 1, plus 25 μg of cycloheximide ml^{-1} as inhibitor of plant and fungal growth.

Each plate was cultured until colonies were large enough for the respective quantitation procedure (see next paragraph). After culture each plate was examined with a dissecting stereomicroscope from the bottom and top. The root zones of seedlings (under agar) were recognized by being stained with congo red. The total length of roots in each group of 15 seedlings, estimated by contour measurements over 18 groups, was quite reproducible (average group length, 23.1 cm; the coefficient of variation [cv] of individual groups, with 95% confidence, was 5.6%). The location of microcolonies in the plate (under 16× or 24× magnification) was noted in relation to each seedling: either in direct physical contact with root surfaces (root hairs or bare epidermis [Fig. 1]), on the shoot surface, or (very rarely) as isolated colonies within the agar medium, visibly separate from plant-agar interfaces. Only colonies in direct contact with the roots were considered to represent genuine root-adsorbed bacteria and were counted as such; colonies on the shoot and isolated colonies were rejected. Care was taken to record colonies observed from both sides of the plate only once.

Quantitative expression and estimation of bacterial adsorption to roots. Adhesiveness (%*A*) is here defined as the percentage of bacteria in the inoculum that under the chosen experimental conditions became adsorbed to roots. The number of root-adsorbed bacteria, detected as colonies in direct contact with the root surfaces, was estimated by either of two procedures.

(i) Direct quantitative procedure. After culturing seedling plates for a limited time (to control colony growth; see below), the total number of microcolonies on root surfaces was directly counted and related to the total number of bacteria initially inoculated to give *A*. The approximate 95% confidence interval for *A* in this procedure is given by the expression $A \pm 1.96A(1/m + 1/n)^{1/2}$, where *m* and *n* are the actual number of colonies counted to measure bacteria adsorbed to the roots and contained in the inoculum, respectively.

In this procedure care was taken to limit the size of microcolonies to about 0.1 mm in diameter (2-day culture for *R. meliloti*), small enough to avoid their merger and keep each microcolony distinct from others on the root surface, at a density of 50 colonies per cm of root length (1,000 to 1,200 per plate, allowing measurements of adsorbed bacteria with 95% confidence limits of ±6%). Counting the colonies was done under 16× to 24× magnification. Both sides of the plate

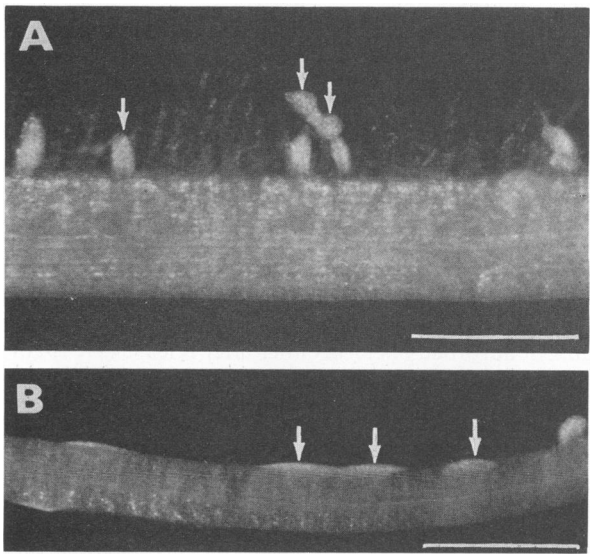

FIG. 1. Appearance of individual microcolonies (arrows) developed on root hairs (A) and on the surface of the younger, hairless region of the root (B). Bar, 500 μm. Alfalfa seedlings with adsorbed *R. meliloti* L5-30 cells were cultured for 2 days embedded in YEM-antibiotic agar (see text).

were counted. Since observation and counting of microcolonies in the agar layer was much easier to perform from the bottom, a comparison was made between total colony counts (t) and bottom counts (b) obtained for individual alfalfa roots over 313 roots in 21 independent experiments. Within a range of colony counts of 1 to 100 per root, the ratio $f = t/b$ was between 1.0 and 1.255. The deviation from the expected ratio of 2 reflects the very frequent observation from the bottom side of colonies on the upper side which were incompletely eclipsed by the root body. A nonlinear relation was observed between f and b, and a quadratic expression could be fitted to the data: $f = 1.064 + (4.304 \times 10^{-3})b - (2.671 \times 10^{-5})b^2$, with a high correlation coefficient, 0.961. This strong correlation allowed us to simplify the colony counting procedure by taking only bottom counts, b, which were used to calculate the respective f with the preceding expression; by applying the correction $f \times b$, the total number of colonies on the roots, t, was estimated.

(ii) Quantal response statistical procedure. Seedling plates were cultured for longer periods (3 days for *R. meliloti*) to ensure macroscopic colony development and easy detection of roots with associated colonies. For each 10-fold dilution of the inoculum (generally five levels were used per assay), roots without visible colonies on their surfaces (negative test units) were scored and their fraction, $\hat{S}$ (relative to the total number of roots present), was recorded. The statistical treatment of Seligman and Mickey (25) or a computational modified Newton-Raphson method for maximum likelihood estimation (24) was applied to these data to estimate the most probable number of bacteria (contained in the nondiluted inoculum) that were able to adsorb to roots. This was related to the total number of viable cells in the nondiluted inoculum to give A. Results of A by this quantal method are here given with an approximate 95% confidence interval, calculated by the expression $A \pm 1.96A(s^2 + 1/n)^{1/2}$, where s is the relative standard error of the estimate of the number of bacteria able to adsorb to roots (25) and n is the actual number of colonies in the plate count assay for viable bacteria in the inoculum. To ensure the validity of this procedure, the assumption that adsorbable bacteria were randomly dispersed throughout the test units was checked by using a chi-square goodness-of-fit test.

All values reported here are given with their 95% confidence intervals. Averages and their confidence limits were obtained by proper weighting of the individual data.

Assay for adsorption of rhizobia to roots as revealed by nodulation. The nodulation assay was performed as in the quantal response statistical procedure. However, bacteria remaining adsorbed to roots after inoculation and standard washing were revealed in this case by nodule formation, and their most-probable-number estimate was obtained from counts of nonnodulated plants. After incubation for bacterial adsorption, the washed, inoculated seedlings (as before, 15 per inoculant dilution level) were mounted individually on sterile filter paper strips, and each was axenically cultured in a capped glass tube (16 by 160 mm) with 3 ml of nitrogen-free Fåhraeus solution in a growth chamber under controlled conditions (26°C in light, 22°C in the dark, with a photoperiod of 16 h). The volume of nutrient in the culture tubes was maintained throughout the experiments by adding sterile water weekly. Plants with and without nodules (positive and negative test units) were scored at day 15 after inoculation. Adhesiveness was calculated from these data by applying the quantal response statistical procedure described above.

TABLE 2. Adsorption of *R. meliloti* L5-30 to alfalfa roots

Procedure	Inoculum (10^3 bacteria per root)	%A[a]	CV(%)[b]	%A (range)
Direct quantitative	1.28–4.68	3.02 ± 0.07 (23)	5–22	2.66–3.33
	0.12–0.51	3.01 ± 0.25 (6)	16–28	2.12–3.59
Quantal response	0.01–0.10[c]	3.03 ± 0.49 (14)	45–74	2.54–3.49

[a] Adhesiveness, weighted average with 95% confidence limits. The number of independent measurements is indicated in parentheses.
[b] Range of CVs (95% confidence) of individual values of A.
[c] Range which yielded roots with and without associated colonies.

TABLE 3. Bacterial adsorption to roots of alfalfa and white clover[a]

Strain	%A	
	On alfalfa roots	On white clover roots
R. meliloti		
L5-30	3.03 ± 0.49 (14)	3.32 ± 1.32 (2)
U45sk	0.50 ± 0.09 (6)	
U210	9.39 ± 3.49 (3)	
R. trifolii A118s	0.62 ± 0.23 (2)	0.12 ± 0.04 (2)
E. coli C74	0.31 ± 0.18 (1)	
B. subtilis		
JA-60-A	0.41 ± 0.42 (2)	
Pb1706	0.49 ± 0.33 (1)	
A. tumefaciens LBA288	7.88 ± 3.12 (2)	

[a] Adhesiveness was measured with the quantal response statistical procedure. Values are weighted averages with 95% confidence limits. The number of independent experiments is indicated in parentheses.

RESULTS AND DISCUSSION

In the present work root-adsorbed bacteria have been defined operationally as bacteria that remain associated with the root surface as a result of incubation of the seedlings with the bacterial inoculum for 4 h in nitrogen-free Fåhraeus solution, followed by four consecutive, controlled washes with fresh medium. Bacteria adsorbed to seedling root surfaces were individually detected as microcolonies which developed after culture of the inoculated seedlings embedded in nutrient agar. Counting the colonies which appeared to be in direct physical contact with the root surface, either directly or by a quantal response method, allowed a quantitative measure of bacterial adsorption to roots.

To be valid, this procedure should exclude from counting any bacteria other than the desired inoculant strain. During preliminary experiments with seedlings from axenically germinated, surface-sterilized (29) alfalfa seeds, noninoculated control roots showed profuse bacterial growth after agar culture. This contamination was caused by an endogenous population of nonnodulating bacteria contained within alfalfa seeds, which—as in other legumes—could not be totally eliminated by usual seed sterilization procedures (1, 13; G. Caetano Anollés, J. Echave, and G. Favelukes, unpublished data). Isolates of endogenous contaminants from alfalfa seeds have been recently identified as *Erwinia herbicola* (15, 16). By extending the seed surface sterilization treatment (as developed by G. Caetano Anollés and J. Echave, see Materials and Methods) beyond standard lengths of time (29), the

number of surface-contaminated, germinated seedlings could be decreased to not more than 4 to 5% of the total plants. The residual contaminants have been found to be sensitive to streptomycin, rifampin, kanamycin, or nalidixic acid. To prevent growth of this population and ensure the selective detection of the inoculant bacteria during colony development, in the present work streptomycin or rifampin was incorporated into the embedding agar medium, and—as a necessary restriction imposed by the method—only antibiotic-resistant bacteria were used as inoculant strains. In this way interference by endogenous contaminants was eliminated, and the development of colonies on alfalfa or clover roots upon culture in embedding agar was consistently dependent on previous inoculation with an antibiotic-resistant strain. Then, colonies in direct contact with the root surface were considered to result from bacteria adsorbed to it.

Figure 1 shows the aspect of microcolonies developed under those conditions on roots of alfalfa seedlings inoculated with *R. meliloti* L5-30 (streptomycin resistant) and cultured for 2 days in streptomycin embedding agar: they were seen as thin white lentils in direct contact with the root surface. On root zones without hairs, colonies sometimes covered the bare epidermis; on hairy zones, microcolonies were often found on hair tips or around their midportions. Occasionally two or three microcolonies could be seen on the same hair.

To study whether every adsorbed bacterium would give rise to an individual colony, different regions along the roots were spot-inoculated with 20 μl of a suspension of *R. meliloti* L5-30 in melted 1% agarose, left to set without rinsing, and cultivated for microcolony development within root-embedding medium containing streptomycin (100 $\mu g \cdot ml^{-1}$). The number of microcolonies per spot (20 ± 1) was compared with that obtained by culturing equivalent spot inocula laid on the embedding agar surface. No statistically significant differences ($P > 0.5$) could be found in the yield of microcolonies per inoculated rhizobium between inocula on roots and on the nutrient medium, a result which argues against root-inhibitory effects on the detection of adsorbed rhizobia as colonies.

In the present work each colony in contact with the root surface was taken to represent a single bacterial cell adsorbed to the root. We believe that this is a reasonable assumption in view of the experimental conditions, especially the high dilution of the inoculum (free of bacterial aggregates) and the sparse distribution of root-adsorbed bacteria imposed by the need to keep neighboring microcolonies separate and distinct. However, in other conditions a colony could represent a set of bacteria adsorbed close to each other, arising as a consequence of collective attraction (a cloud or swarm), from a bacterial aggregate, or as a localized multiplication of already bound bacteria. The magnitude of these phenomena may be assumed to increase with bacterial concentration and with time. Particularly, their observation by direct microscopy recorded in the literature (8–10, 12, 15) has always been obtained with concentrated inocula.

After culturing embedded, inoculated seedlings, very few if any bacterial colonies (never exceeding 1% of the total colonies in the petri dish) appeared in the agar medium isolated from direct contact with the root surfaces: these colonies were excluded from counting. Presumably they developed from bacteria released from the roots during the embedding operation. The small proportion of isolated microcolonies indicates that the agar embedding procedure preserves efficiently the original location of the root-adsorbed bacteria and points to the considerable physical stability of these associations.

Whole seedlings were used throughout this work instead of excised roots to avoid any possible disturbing effects upon bacterial adsorption by tissue components leaking through the wound to the medium.

Two approaches were used to evaluate the number of adsorbed bacteria. In the first, microcolonies developed along the roots were directly counted. This direct quantitative procedure accommodated a wide range of adsorbed bacteria; the approximate upper limit for the observation of separate microcolonies (referred to above) was 50 adsorbed bacteria per cm of root length. The second approach was a quantal response statistical procedure similar to those used for counting bacteria, viruses, and bacteriophages (21). In this case, each set of adsorption experiments was carried out with increasing numbers of inoculant bacteria, and colonies were overgrown to ensure their easy detection. By scoring roots devoid of associated colonies, the most probable number of bacteria able to adsorb to roots was obtained. Appropriate sets of 10-fold bacterial dilutions gave estimates of bacterial adsorption which generally fell within the optimal range of the quantal procedure, 1.1 to 2.3 adsorbed bacteria per root (25). Adsorption in such small numbers allowed the use of extremely dilute inocula.

The results obtained by either counting method were compared with the total number of bacteria in the respective inoculum, allowing calculation of the percentage of bacterial adsorption to roots, here called adhesiveness (*A*; see Materials and Methods).

As a test of both procedures, the adsorption of *R. meliloti* L5-30 to alfalfa roots was measured many times under the standard conditions described in Materials and Methods. In a series of 29 independent experiments with the direct quantitative procedure, groups of 15 plants were inoculated with 0.1×10^3 to 4.7×10^3 rhizobia per root. With the quantal procedure, in a separate series of 14 independent assays, groups of 15 plants were treated with each of five 10-fold dilutions of rhizobia (ending at about 1 or less inoculant rhizobium per root). As expected from the little information handled in the quantal approach, the precision of *A* obtained by this method compared unfavorably with the results derived from direct counting (Table 2). However, the remarkable coincidence in the average value of *A*, 3.0%, obtained by either method signals the inherent consistency of both procedures. Thus, the degree of adsorption of a bacterial strain to roots can be measured reliably even at levels as low as 1 adsorbed bacterium per root.

The reproducibility of adsorption was found to depend on careful standardization of conditions such as the state of the inoculant bacteria and the seedlings, the incubating medium, and the incubation period (G. Caetano Anollés and G. Favelukes, Abstr. 3rd Int. Symp. Microb. Ecol., 1983, E-11, p. 39), as well as the rate of circular shaking during incubation (optimal at about 50 rpm; 0 or 100 rpm caused decreases of 70 and 83%, respectively) and the root washing procedure that followed. The 4-h period of incubation for adsorption was chosen to minimize secondary phenomena such as bacterial proliferation and extensive rhizosphere colonization or phase 2-like binding of rhizobia to roots (12) while still allowing a sizable degree of adsorption (more than 10 times the degree of adsorption obtained at 1 h).

The adsorption of various bacterial strains to alfalfa and white clover roots was compared under the standard conditions (Table 3). When confronted with their symbiotically

homologous alfalfa host, three strains of *R. meliloti* showed widely different degrees of adsorption, ranging over a 20-fold span. *R. trifolii* A118s (heterologous) was comparable to the least adsorptive *R. meliloti* strain. Similar levels of adhesiveness were also shown by *E. coli* and *B. subtilis*. One strain of *A. tumefaciens* (nonsymbiotic) was highly adsorptive, second only to the most adsorptive strain of *R. meliloti*. The adsorption of *R. meliloti* L5-30 and *R. trifolii* A118s was also studied on white clover roots: *R. trifolii* (but not *R. meliloti*) was symbiotically homologous. Despite this permutation in homology, the relative levels of adsorption of both strains in alfalfa roots were not reversed in white clover. These results indicate that (i) diverse rhizobial strains, all symbiotically competent for a legume, may behave very differently during root adsorption and (ii) total adsorption of bacteria to the overall surface of legume roots does not reflect the specificity of the process of infection and nodulation. However, since the present method does not allow the determination, with high microscopic resolution, of the particular site on the root where each adsorbed bacterium is located, it cannot be ruled out that adsorption to specialized locations on the alfalfa roots (as has been shown for adsorption to root hairs in other legumes; cf. references 11, 12, 18, and 27) might in turn show the selectivity of the symbiotic association. In fact, an indirect demonstration that the phenomenon of specific adsorption does occur in alfalfa is given in the accompanying paper (5).

The experiments included in Table 2 provided additional information about some properties of the adsorption of *R. meliloti* to alfalfa roots in mineral solution. The constancy of A (Table 2) measured by either quantitation procedure in a range of two decades of L5-30 inoculum, from 10^1 to 10^2 (quantal response) to 10^2 to 10^3 (direct count) bacteria per root, indicates that within this interval, bacterial adsorption is proportional to the inoculum size and that large increases in the bacterial inoculum would be required to reach root saturation. Due to the limitations in the density of adsorbed bacteria resolved by colony counting, the upper ranges of the inoculum dose-adsorption curve have not been studied. The 14 independent quantal response experiments quoted in Table 2 were analyzed further by means of a log-log (Weibull) plot (see reference 21). A straight line was obtained by linear regression analysis [$\log(-\log\hat{S}) = (0.63 \pm 0.06)\log B - 1.05$, where $\hat{S}$ is the fraction of roots devoid of colonies in each incubation and B is the number of bacteria inoculated per root; $r = 0.88$, $t(27) = 9.63$, $P < 0.005$], which was displaced and different in slope from that expected for a purely Poissonian distribution of the bacteria on the roots; this departure from such simple behavior indicates a source of heterogeneity in the system (21), either in binding sites on the roots, in adsorbing bacteria, or in both.

From data obtained with the direct method, we examined the distribution of L5-30 rhizobia on 60 alfalfa seedlings. Adsorption on the shoots was minimal, only 8.7 ± 0.3% of the total bound. The high proportion of bacteria associated with the roots indicates the high adsorptive activity of these for rhizobia, probably stimulated by chemoattraction by root exudates (cf. Gulash et al. [15]). Rhizobia appeared to be evenly distributed on the roots, with 33.3 ± 4.3, 29.6 ± 3.4, and 37.6 ± 4.4% associated with the apical, middle, and proximal thirds of the root, respectively. These zones correspond, in that order, to increasing age and degree of maturity. Bhuvaneswari et al. (2), working with larger alfalfa roots, found marked differences in the susceptibility of different zones of the root for infection and nodule formation; it was maximal at the developing root hair zone near the apex. In our case, the lack of differential behavior for adsorption along the roots could be due to the limited development and immaturity of our 5-day-old seedlings.

Although the procedures described here were devised to handle small-rooted plants, they might conceivably be extended to roots of larger sizes by suitable modifications, allowing dilute inocula to be used and adsorbed bacteria to be located along the root surface.

A modification of the quantal response procedure was devised to quantitate solely those adsorbed rhizobia that actually formed a nodule. In this approach, root-adsorbed *R. meliloti* L5-30 cells arising from a regular incubation of 15 seedlings with inoculum followed by four washes, as described in Materials and Methods, instead of being cultured for colony development, were subsequently allowed to infect and nodulate their supporting alfalfa roots during axenic growth of each plant separately in Fåhraeus solution. Rhizobial adsorption was thus revealed by nodule formation. Since only the inoculant strain was competent for nodulation and contaminating bacteria from seeds did not interfere with this assay, the use of antibiotics was not necessary. Thus, the modified procedure allowed the assay of adsorption of antibiotic-sensitive infective rhizobia. The basic assumption was that, at high dilution, nodulation would be the result of infection by at least one rhizobial cell initially adsorbed to the root. The proportion of nonnodulated roots for each dilution of the inoculant was used to estimate the most probable number of bacteria capable of root adsorption followed by nodulation. In this way we obtained $A_{nod} = 0.78 \pm 0.23\%$ (average of two experiments). Compared with values of A obtained from colony-development quantal assays (Table 2), this result suggests that at these very low levels of adsorption, at least one of every four adsorbed L5-30 rhizobia succeeded in the process of root infection and nodulation. Under our conditions, then, at least a substantial fraction of the adsorbed *R. meliloti* cells may have represented functional precursors of the infection process. The low yield of nodulation per adsorbed *R. meliloti* cell may reflect a heterogeneity of binding sites on the roots (such as that previously mentioned), some of which might be nonspecific (5) or infertile for nodule development (2, 3, 28), as well as the existence of infection and nodulation processes aborting at intermediate stages (7).

Quantitative studies of bacterial adsorption to root surfaces reported by several authors (referred to in the Introduction) have generally dealt with large numbers of attached bacteria (usually no less than 10^4 rhizobia per cm of root length), and the respective concentrations of free bacteria (in the inoculum or established in the vicinity of the roots) were also high, on the order of 10^6 or more ml^{-1}. While these numbers are not far from the concentrations found in well-established bacterial populations in the rhizosphere, they do not reflect the situation in many soils with lower rhizobial concentrations, not infrequently in the order of 10^2 to 10^4 g^{-1} of soil. At the rapid rate of elongation of growing rootlets in young seedlings, newly developed portions close to the root tip which are maximally active for nodule initiation in several legumes (2, 3) would be exposed to fresh sections of the soil, where the existing population of rhizobia would have been subjected to localized rhizosphere stimulation and attraction (15) by root exudates, only for limited periods of time. Then it might be expected that early interaction of young roots with rhizobia would have taken place at low bacterial concentrations. In the method reported here, rhizobial numbers in the inoculum were small (from 1 to 10^5 ml^{-1}), thus covering the lower end of the concentration

spectrum likely to operate during early adsorption to roots in the soil. In a recent study of bacterial adsorption to soybean root tips, Pueppke (22) also used dilute (10^4 organisms ml^{-1}) inocula.

The present method has been found to be advantageous in the following aspects. (i) The procedure is simple and amenable to most microbiological laboratories. (ii) It uses low bacterial concentrations, reflecting those found in sparsely populated soils. (iii) Macroscopic localization and frequency of distribution of adsorbed bacteria along the root can be easily observed and related to the anatomical features of the host. (iv) In principle, it could be used with any pair of small-rooted plant and bacteria (i.e., symbiotic, associative, or pathogenic) provided that the former is free of bacterial contaminants or that the microorganism is antibiotic resistant. (v) It can be used to estimate the fraction of adsorbed rhizobia which result in nodule formation. (vi) Adsorption of an antibiotic-resistant strain can be assayed in the presence of any sensitive bacteria (even in the case of endogenous root contaminants), however complex this population may be. In particular, this would allow the study of adsorption under conditions of competition or antagonism by other strains. This feature has been exploited in the accompanying paper (5), where competition for adsorption of *R. meliloti* by homologous and heterologous rhizobia and other bacteria allowed detection and quantitation of symbiotic-specific and nonspecific adsorption to alfalfa roots.

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