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Role of Motility and Chemotaxis in Efficiency of Nodulation by *Rhizobium meliloti*[1]

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GUSTAVO CAETANO-ANOLLÉS*[2], LUIS G. WALL, ANA T. DE MICHELI, EDGARDO M. MACCHI, WOLFGANG D. BAUER, AND GABRIEL FAVELUKES
Cáteda de Química Biológica I (G.C., L.G.W., A.T.D., G.F.) *and Instituto de Investigaciones Fisicoquímicas Teoricas y Aplicadas* (E.M.M.), *Facultad de Ciencias Exactas, Universidad Nacional de la Plata, 1900-La Plata, Argentina; and Department of Agronomy, Ohio State University, Columbus, Ohio 43210* (W.D.B.)

ABSTRACT

Spontaneous mutants of *Rhizobium meliloti* L5-30 defective in motility or chemotaxis were isolated and compared against the parent with respect to symbiotic competence. Each of the mutants was able to generate normal nodules on the host plant alfalfa (*Medicago sativa*), but had slightly delayed nodule formation, diminished nodulation in the initially susceptible region of the host root, and relatively low representation in nodules following co-inoculation with equal numbers of the parent. When inoculated in growth pouches with increasing dosages of the parental strain, the number of nodules formed in the initially susceptible region of the root increased sigmoidally, with an optimum concentration of about 10^5 to 10^6 bacteria/plant. The dose-response behavior of the nonmotile and nonchemotactic mutants was similar, but they required 10- to 30-fold higher concentrations of bacteria to generate the same number of nodules. The distribution frequencies of nodules at different positions along the primary root were very similar for the mutants and parent, indicating that reduced nodulation by the mutants in dose-response experiments probably reflects reduced efficiency of nodule initiation rather than developmentally delayed nodule initiation. The number of bacteria that firmly adsorbed to the host root surface during several hours of incubation was 5- to 20-fold greater for the parent than the mutants. The mutants were also somewhat less effective than their parent as competitors in root adsorption assays. It appears that motility and chemotaxis are quantitatively important traits that facilitate the initial contact and adsorption of symbiotic rhizobia to the host root surface, increase the efficiency of nodule initiation, and increase the rate of infection development.

Soil bacteria belonging to the genus *Rhizobium* induce the formation of symbiotic nitrogen-fixing nodules on the roots of leguminous plants. The establishment of an effective nodule occurs through a complex sequence of interactions which may begin even prior to physical contact between the partners (4, 5, 29). Rhizobia are initially attracted to the developing roots, where they adsorb to the root surface and elicit root hair deformation, cortical cell division and the formation of an infection thread which carries the bacterial symbiont into the root cortex.

The role of bacterial motility and chemotaxis in this process is not well defined. Previous studies have shown that behavioral mutants of *R. meliloti* are able to induce the formation of normal nodules on the host plant alfalfa, indicating that motility and chemotaxis are not essential to the infection and nodulation processes (3, 30). However, there is evidence to show that motility and chemotaxis can confer a significant advantage during infection and nodulation. Ames and Bergman (2) reported that the motile, chemotactic wild-type strain was capable of forming between 65 and 98% of the nodules when competing against equal numbers of nonflagellated or nonmotile mutant isolates. The basis of the competitive advantage enjoyed by the motile, chemotactic parental cells in these experiments has been somewhat uncertain, particularly since very high inoculum concentrations were used to minimize subsequent bacterial multiplication. Chemotaxis and motility might have provided enhanced chemical or physical contact with the root, enhanced occupation of sites potentially suitable for infection (22), and/or more rapid or efficient infection development. The present studies seek to improve our understanding of these possibilities. Several behavioral mutants and their parent have been compared quantitatively with respect to relative adsorption to the host root surface, ability to inhibit adsorption of competing bacteria, inoculum dose/nodulation response behavior, and nodule occupancy following mixed inoculations. (A preliminary account of part of these studies was presented to the 6th International Symposium of Nitrogen Fixation, Corvallis, OR, August 4–10, 1985.)

MATERIALS AND METHODS

Rhizobium Cultures and Plant Material. Mutant strains used in this study are listed in Table I. *Rhizobium meliloti* strain L5-30 was obtained from G. Martínez-Drets, Montevideo, Uruguay, strain U45 from M. Barate de Bertalmío, Montevideo, Uruguay, and *R. trifolii* A118 from INTA, Castelar, Argentina (original strain, TAI, CSIRO, Australia). *R. meliloti* L5-30-1 is a rifampin-resistant derivative of strain L5-30 which was found to nodulate as well as the parent. *Medicago sativa* cv Dawson was supplied by Alexandre & Cia., Buenos Aires, and cv Vernal by Dewine & Hamma Seed Co., Yellow Springs, OH. General procedures for maintenance of stock cultures, initiation of starter cultures, and preparation of inocula have been already described (11). Seeds were surface sterilized and germinated on water-agar plates (11).

Isolation of Behavioral Mutants. Spontaneous nonmotile and defectively motile mutant strains of *R. meliloti* strains L5-30 and U45 were isolated by enriching for bacteria which did not migrate in semisoft agar as described by Napoli and Albersheim (30).

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[2] Present address: Agronomy Department, Ohio State University, Columbus, OH 43210.

Table I. *R. meliloti Isolates and Their Characteristics*

Isolate	Swimming Behavior[a]	Bacteria/ Capillary	Chemotactic Ratio[b]	Swarm Size[c]	Flagella[d]	Phenotype
				cm		
L5-30	Fully motile	746 ± 178	9.0	2.8	+	Wild Type
LP302	Incessant tumbler	1802 ± 915	0.6	0.2	+	Che$^-$
LP304	Incessant tumbler	ND	ND	1.0	+	Che$^-$
LP206	Nonmotile	61 ± 33	2.8	0.2	+	Mot$^-$
LP209	Nonmotile	ND	ND	0.3	+	Mot$^-$
LP101	Nonmotile	98 ± 56	0.9	0.8	−	Fla$^-$
LP107	Nonmotile	ND	ND	0.5[e]	−	Fla$^-$

[a] Assessed by direct microscopy. [b] Ratio between bacteria in capillary with and without attractant (2 mM proline). [c] In mannitol-glutamate swarm plates after 48 h of growth. [d] Flagella were detected as described in "Materials and Methods." [e] Peripheral microcolonies around the point of inoculation.

Bacteria from stationary phase cultures were picked onto swarm plates containing 35 to 40 ml of semisoft (0.3%) agar mannitol-glutamate medium (8). Seven days after inoculation, material from the center of the swarms was again picked onto fresh semisolid plates. This enrichment procedure was repeated seven times. Individual isolates were then screened for motility on swarm plates. If their behavior was abnormal, they were further characterized. To confirm that mutants were isogenic derivatives of the parent strain, both parent and derivative bacteria were typed for sensitivity to phages N3, N9, and M4 and resistance to phages 43 and A3 (obtained from G. Zannassi, La Plata; and S. M. Lesley, Agriculture Canada, Ottawa, Ontario). The growth rate of the different mutants in liquid culture was found to be comparable to that of the parent. All behavioral mutants were checked repeatedly for phenotypic stability on swarm plates over a 2 year period.

Bacterial Flagella. The presence and distribution of flagella was examined by transmission electron microscopy. Portions of bacterial cultures were grown to the desired density in YEM[3] (11) or mannitol-glutamate broth (8) at 30°C and 150 rpm in a rotary water-bath shaker and then placed onto irradiated carbon-coated parlodion membrane copper grids for 20 min, blotted dry, washed twice with sterile water, negatively stained with 1% uranyl acetate for 1 min, again blotted, and finally allowed to air dry. Specimens were observed with a Siemens Elmiskop 101,I/IA,II with an acceleration voltage of 60 kV. At least 100 cells were examined in each preparation to estimate the number and distribution of flagella. Cultures in late exponential phase were also examined by light microscopy using the flagella staining methods of Gray (17). Cells were washed, resuspended in 10% (v/v) formalin, and stained in the presence of the mordent for 6 min.

Motility and Chemotaxis Assays. Motility and chemotaxis were studied using Pffefer's capillary tube assay as modified by Adler (1). Bacteria were grown in Götz medium (21) to late exponential phase. The cells were separated from the culture medium by centrifugation at low speed (1000*g*, 10 min) at room temperature and then resuspended in buffer (1) to approximately 10^8 bacteria/ml. The bacterial suspensions were placed in chambers of 0.2 ml capacity constructed from two microscope slides separated by a U-shaped Teflon plate 1 mm wide. Two l-μl capillary tubes (Microcaps, Drummond Sci. Co., Broomall, PA) containing buffer, 2 mM proline, or 250 mM aspartate in buffer were sealed on one side, and after 10 min introduced into each chamber and incubated horizontally at 30°C for 1 h. The number of bacteria that entered the capillary tubes was determined by plating appropriate dilutions onto YEM agar plates. The mean accumulation in capillary tubes from triplicate assays was expressed as total cells per capillary, and the chemotactic response from 2 to 3 independent experiments was determined as the ratio of bacterial accumulation in capillary tubes with or without attractants. Suitable attractants and concentrations were taken from Burg *et al.* (9). Motility was also examined by light microscopy. Several fields of bacteria were examined after appropriate dilution of the original suspensions. As controls for these microscopic observations, nonmotile bacteria were prepared by pretreating cultures with 8 mM NaN_3.

Adsorption of Bacteria to Roots. Root-adsorbed rhizobia were quantitatively determined as described previously (11). Fifteen 5-d-old seedlings were incubated (50 rpm, 28°C) for 4 h in 22.5 ml Fahraeus mineral solution (18) containing 10^3 to 10^4 bacteria/ml, followed by four consecutive 25 ml washings with fresh mineral solution. Adsorbed bacteria were individually detected and counted along each root as microcolonies which developed upon culture of the washed seedlings after embedding in YEM agar as previously described (11). Adsorption is expressed here in terms of 'adhesiveness,' which is defined as the percentage of *Rhizobium* cells that became adsorbed under the chosen experimental conditions (11). In competition experiments, adsorption of antibiotic-resistant bacteria to alfalfa roots was studied in the absence or presence of the antibiotic-sensitive competitor bacteria, with addition of the appropriate antibiotic to the embedding medium.

In preliminary tests, *R. meliloti* was found to adsorb to alfalfa roots at comparable levels with either cv Dawson or cv Vernal. The root length of 5-d-old plants of the two cultivars was also comparable (2.0 ± 0.6 and 2.2 ± 0.6 cm (SD), respectively). Adsorption was optimal when late exponential phase bacteria were used.

Nodulation Efficiency of Rhizobia. Seedlings 2 d old were transferred from water-agar plates to plastic growth pouches (Northrup King Seed Co., Minneapolis, MN), five per pouch, moistened with 10 ml of nitrogen-free Jensen plant mineral solution (27). Roots of individual 5-d-old seedlings were inoculated with 100 μl of an appropriate dilution of the bacterial suspension by dripping the suspension onto the root from the tip towards the base. The positions of the RT and of the EH at the time of inoculation were determined by inspection under a dissecting microscope and marked on the plastic face of the pouch as described (6). Plants were cultured in a growth chamber at 70% RH, 28°C in the light, 24°C in the dark, with a photoperiod of 16 h and a light intensity of approximately 1.7×10^4 lux. The number and relative location of nodules on the tap root was determined 8 d after inoculation, using an Apple graphics tablet with digitizing pen (Apple Computer Inc., Cupertino, CA) coupled to an Apple IIe computer and a suitable program (7). Half-strength nitrogen-free Jensen solution was used to moisten the pouches as required. Experiments were repeated at least twice with at least 100 replicate plants used for each treatment.

[3] Abbreviations: YEM, yeast extract mannitol; EH, smallest emergent root hair; RT, root tip.

To determine the relative number of bacteria in pouches with or without plants, immediately after inoculation or 12 h later, strips 2-cm high were cut across the width of each paper towel approximately 1 cm below the seed trough, placed two per tube in 4 ml of Hoagland solution (26) containing Tween 20 (1 drop/100 ml), and sonicated with a Heat Systems sonicator (model W370, Farmingdale, NY) for 5 min at 50% power. Appropriate aliquots of the solution were then plated on YEM agar plates. The pouches without plants were inoculated by dripping the bacteria onto the towel, upwards from an imaginary RT mark located 3 cm from the trough. If pouches contained plants, care was taken to cut a paper strip that covered the last 2 cm of the roots. The 2-cm root segments were pooled and sonicated separately in 2 ml of solution. Approximately 50% of the total added bacteria at the time of inoculation were recovered on the paper strips. The ratio between final and initial bacterial counts were compared for each strain by ANOVA using data from three replicates per treatment and two independent experiments.

Nodulation Assays. Two-d-old alfalfa seedlings from surface-sterilized seeds were transferred aseptically to 250 ml beakers with glass covers containing a wire grid to secure the hypocotyls with their roots immersed in Rigaud and Puppo mineral solution (33) and cultured in a growth chamber under controlled conditions (26°C in light, 22°C in the dark, with a photoperiod of 16 h). Seedlings were also cultured individually on paper strips in 16 × 160 mm capped culture tubes with 3 ml of Rigaud and Puppo or Fahraeus (18) solution, in groups of five on paper strips in 20 × 250 mm capped glass tubes containing 10 ml of Fahraeus solution, or in 800 ml capacity glass pots with covers containing 100 g of sterile vermiculite and 50 ml of plant mineral solution (25). The seedlings were generally inoculated at day 5 with 10^3 to 10^4 total rhizobia.

Recovery of Bacteria from Nodules. Nodules were removed from the plants aseptically, surface sterilized (1 min in 0.2% $HgCl_2$-0.5% HCl, and four washes with sterile water), placed individually in 96-well microtiter plates with 0.1 ml Hoagland solution (26), and finally crushed with a glass rod to release their contents. In some cases the contents of each nodule were assayed *en masse* for swarm morphology, and in others only individual clones were tested.

RESULTS

Microscopic Observations. The swimming behavior of *R. meliloti* L5-30 was studied in liquid media by direct microscopy and the presence, distribution, and structure of flagella examined by transmission electron microscopy. Motility consisted of straight-line bacterial runs interspersed with brief stops or tumbling periods. Light microscopic observations of live bacteria indicated that approximately 60% of the bacterial cells in exponential phase cultures grown on the YEM medium were motile, while 35 ± 9% of the cells in these cultures lacked flagella. Fewer of the cells from stationary phase cultures appeared to be motile and the cells were mainly nonflagellated (65 ± 4%). *R. meliloti* L5-30 possessed up to 5 peritrichous flagella, generally with one flagellum inserted subpolarly. Bacteria with fewer flagella usually showed subpolar flagellation. It was common to observe cells with one subpolar flagellum and several broken peritrichous stumps. These observations suggest that subpolarly inserted flagella are more resistent to rupture by manipulation, which is consistent with observations in other fast-growing rhizobia (16). Flagellar filaments presented a clear zig-zag configuration of high electron density bands around a possible central filament and a total diameter of 16.0 ± 0.9 nm. These observations are consistent with those obtained by Götz *et al.* (21) for *R. lupini* H13-3 and *R. meliloti* MVII-1 and suggest that *R. meliloti* L5-30 has complex flagellar filaments. No extracellular structures other than these complex flagella were detected.

Isolation and Initial Characterization of Behavioral Mutants. Spontaneous nonmotile and defectively motile mutants of *R. meliloti* were isolated by enriching for bacteria which did not migrate in semisoft agar. As shown in Table I, isolates representing the three classes of behavioral mutants described by Ames *et al.* (3) were used for further study: mutant LP101 which lacked flagella (Fla$^-$); mutant LP206 with inactive flagella (Mot$^-$); and mutant LP302 with motile but generally nonchemotactic cells (Che$^-$). In addition to these mutant isolates, several other behavioral mutants were isolated which either formed small swarms or developed small peripheral colonies near the point of inoculation (Table I). These swarm morphologies could not be assigned to any of the three classes of mutants described above.

All of the Che$^-$, Mot$^-$, and Fla$^-$ mutants tested in our studies were able to form functional nodules on alfalfa cv Vernal and Dawson regardless of whether the plants were grown in liquid culture, in plastic growth pouches, or in closed glass containers filled with vermiculite or exposed to different plant nutrient solutions. When rhizobia were recovered from the nodules, only the inoculant bacterial phenotype could be identified among the isolated colonies. This was taken to indicate that the mutant strains themselves and not revertants were responsible for the nodules produced.

Rate of Appearance of Nodules on Alfalfa Plants Inoculated with Behavioral Mutants. When alfalfa seedlings were grown on filter paper strips in test tubes and nodulation was examined at daily intervals, nodules began to appear on the primary roots about 4 d after inoculation with the parent. Between 4 and 10 d after inoculation, the rate of appearance of nodules was considerably lower for the Che$^-$ (LP302) and Fla$^-$ (LP101) mutants than for the parent and was somewhat lower for the Mot$^-$ mutant, LP206 (Fig. 1). However, by 10 to 12 d after inoculation, overall rate of nodule appearance increased until it was the same for the mutants as for the parent. The overall effect of these mutations was to delay the appearance of nodules by approxi-

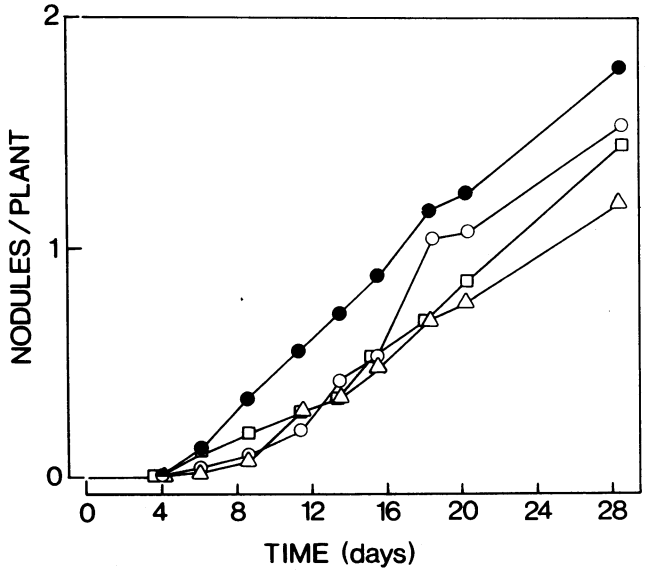

FIG. 1. Time course of nodulation in alfalfa by *R. meliloti* strains L5-30 (●), LP302 (○), LP206 (□), and LP101 (△). Fifty groups of five 5-d old seedlings were inoculated with 10^5 bacteria/ml and grown in Fahraeus mineral solution as described in "Materials and Methods." Plants were examined at daily intervals and the number of nodules per plant determined. Each point represents a weighted average of results from one of two independent experiments. There was less than 5% variation between individual replicates.

mately 4 d. Nodulation was similarly delayed relative to controls with the parent when plants were grown in vermiculite and inoculated with LP101 (data not shown).

Nodulation in the Initially Susceptible Region of the Root. As shown in Figure 2, few if any nodules developed in the region of the primary root where mature, fully elongated root hairs were present at the time of inoculation with the wildtype isolate, *i.e.* in the region more than about three relative distance units above the RT. A relative distance unit is defined as the distance between the root tip and the smallest emergent root hairs on a given plant at the time of inoculation (6). This developmentally restricted pattern of nodulation is in agreement with previous observations with other isolates (6) and suggests that the initiation of nodules on alfalfa roots by *R. meliloti* L5-30 takes places only in the zone of emerging root hairs. Inspection of the nodulation profiles in Figure 2 reveals that nodules were formed at similar frequencies in various regions of the primary root regardless of whether they were inoculated with the parent L5-30 or with the mutants. The median location of nodules on the primary root was calculated in order to indicate possible differences in nodule distribution between mutants and parent. The average location of the median nodule position from three independent experiments was determined to be 0.57 ± 0.36, 0.53 ± 0.33, 0.55 ± 0.32, and 0.70 ± 0.34 relative distance units below the RT mark for strains L5-30, LP302, LP206, and LP101, respectively.

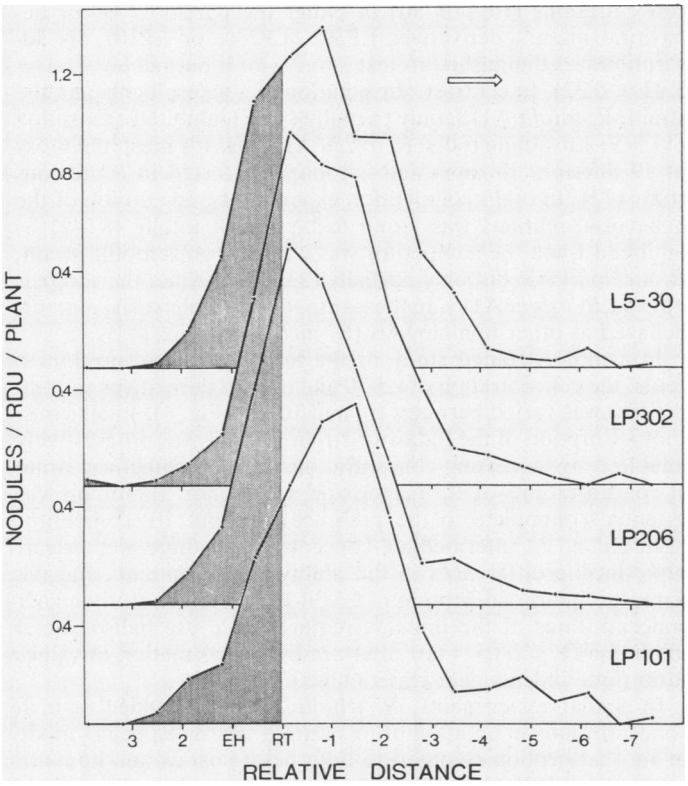

FIG. 2. Profiles of nodulation frequency for alfalfa cv Vernal inoculated with *R. meliloti* L5-30 and mutant derivatives. Plants were inoculated at d 5 with 2.1 to 2.8 × 10^4 bacteria/plant and nodules scored 1 week later. No nodules were observed on uninoculated control plants. The position of nodules on the primary root of 120 to 140 plants were measured (±0.5 mm) relative to the RT mark. The relative distance of each nodule on the primary root from the RT mark was expressed as a percentage of the RT to EH distance determined for each plant. The average RT-EH distance for 1235 inoculated plants was 5.02 ± 0.28 mm (SD). Since the average rate of root elongation was 0.42 ± 0.03 mm/h, one relative distance unit (RDU) is equivalent to approximately 12 h of growth. The shaded areas indicate nodulation in the initially susceptible region above the RT mark. The direction of root growth is shown by the arrow.

A more detailed analysis of nodule initiation revealed that the parent consistently generated more nodules in the initially susceptible region of the root above the RT mark than any of the three behavioral mutants (Table II). These differences in frequency of nodule initiation were generally less than twofold, but statistically significant ($P < 0.05$). No significant differences were found in the total number of nodules formed on the primary roots by the mutants and parent (Table II).

The relative efficiencies of nodule initiation by the parent and the behavioral mutants was determined by comparing the dependence of nodule formation above the RT mark on inoculum dosage (Fig. 3). At relatively low inoculum dosages of the parental strain, the slope of the nodulation response curve was quite low. However, at dosages of about $10^{3.5}$, the slope of the curve increased abruptly. Similar dose-response behavior has recently been observed for another wildtype strain, *R. meliloti* RCR2011 and appears to indicate the existence of a threshold of response in the host root to bacterial signals (10). At dosages of about 10^6 L5-30 cells/plant, the number of nodules generated above the RT mark reached a maximum and then slightly but consistently declined at higher inoculum dosages. The basis for this diminished efficiency of nodule formation at high dosages is not known. The dose-response curves for the mutants were generally similar in shape to the parental dose-response curve but were displaced towards higher dosages. About 10 to 30 times more cells of the mutants were required to elicit half-maximal nodulation. Two of the behavioral mutants did not elicit reduced nodulation at high inoculum dosages (Fig. 3).

Examination of nodulation profiles from the dose-response studies in Figure 3 (data not shown) revealed that the peak of greatest frequency of nodule formation shifted acropetally when the inoculum dosage was increased from 10^2 to 10^7 bacteria/plant, thereby increasing the proportion of nodules above the RT mark. Thus, differences in the average number of nodules formed above the RT mark should be interpreted in terms of nodulation efficiency only where the inoculum dosage has been kept constant, as in Table II. The basis for this acropetal shift in nodule distribution is not known (10).

Bacterial Multiplication after Inoculation. The number of cells of L5-30 was found to increase 1.84 ± 0.12 and 1.78 ± 0.42 times during a 12 h period after inoculation in pouches with or without plants, respectively. The mutant derivatives increased similarly (1.7- to 1.9-fold). No statistically significant differences in multiplication were found between mutant strains, between mutants and L5-30, or between pouches with or without plants ($P > 0.05$). Thus, there is no evidence for differential multiplication of the parent and mutant strains under the conditions used for most of the nodulation assays.

Nodule Occupancy following Inoculation with a Mixture of Parental and Mutant Bacteria. When inoculated in growth pouches with mixtures containing equal numbers of the parent and a mutant derivative, the percentage of nodules above the RT mark occupied by the parent strain was 3- to 10-fold higher than for the mutants (Table III). Nodules containing both parent and mutant were rare.

Effects of Centrifugation of Bacteria on Motility and Adsorption to Roots. In many previous studies, attachment of bacteria to roots has been assayed after centrifugation of the original cultures. We have found that centrifugation (10,000*g*) and gentle resuspension caused significantly diminished motility and root adsorption. Immediately after centrifugation and resuspension in chemotaxis buffer, the motility of L5-30 was reduced by a factor of about 120, as measured by the reduced number of bacteria (9.0 ± 0.5) that were able to enter a buffer-filled cap-

Table II. *Relative Nodulating Ability of R. meliloti L5-30 and Mutant Derivatives*

Plants were inoculated with 2.1 to 2.9 × 10^4 bacteria/plant and nodules from 94 to 138 plants scored 1 week later. Values are averages of two independent experiments.

Strain	Phenotype	Average Number of Nodules above RT	Total Nodules/Root	Plants Nodulated in Marked Root Zone[a]	
				NRH	DRH + MRH
				%	
L5-30	Wild type	0.85 ± 0.17[b]	4.64 ± 0.32	62	25
LP302	Che⁻	0.53* ± 0.13	3.92 ± 0.31	46	18
LP206	Mot⁻	0.45* ± 0.13	3.78 ± 0.29	54	21
LP101	Fla⁻	0.47* ± 0.14	3.96 ± 0.30	44	21

[a] NRH: no root hair; DRH: developing root hair; MRH: mature root hair. [b] 95% confidence interval. * Significant differences ($P < 0.05$; ANOVA test after square-root transformation).

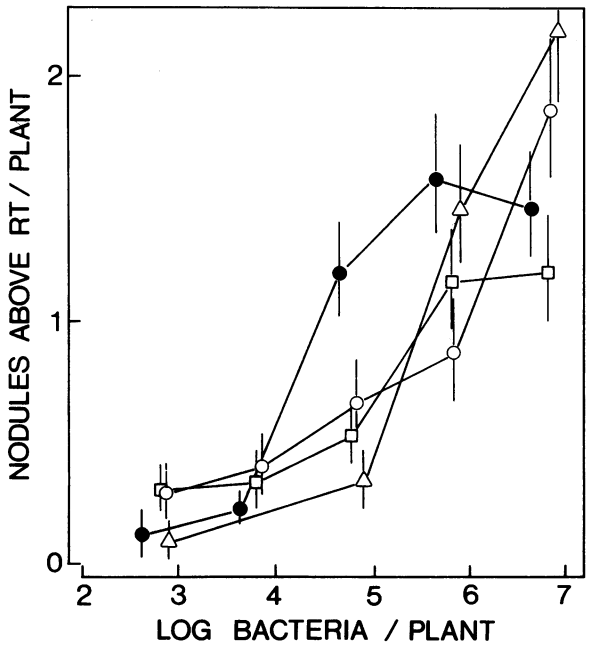

FIG. 3. Dose-response behavior in nodulation of alfalfa cv Dawson by *R. meliloti* L5-30 (●) and the behavioral mutant derivatives LP302 (○), LP206 (□), and LP101 (△). Inocula were prepared by 10-fold serial dilutions of late exponential cultures. Sets of 40 to 50 plants were inoculated per treatment, in three independent experiments. Vertical bars indicate 95% confidence intervals for this representative experiment.

illary tube in a period of an hour compared to an average of about 750 bacteria/tube with uncentrifuged suspensions (Table I). This diminished motility could result from removal and breakage of flagella, since both the percentage of flagellated cells and the number of intact flagella per cell were markedly reduced after centrifugation. When centrifuged and resuspended cells of L5-30 were assayed for root attachment, the fraction of suspended bacteria that adsorbed to alfalfa roots during a 4-h incubation was only 1.9 ± 0.2%, as compared with 3.0 ± 0.3% of the cells from uncentrifuged suspensions. Thus, root adsorption was significantly affected by prior centrifugation even though new flagella were probably formed early during the incubation period. Bacteria resuspended after filtration of cultures through 0.2 μ membranes showed similarly diminished root adsorption.

Effects of Motility and Chemotaxis on Adsorption to Host Roots. The behavioral mutants adsorbed to alfalfa roots in considerably lower numbers compared to the parent L5-30 (Table IV). The Che⁻ mutants LP302 and LP304 adsorbed 16 to 19% as well as the parent while the Fla⁻ and Mot⁻ mutants adsorbed only 6 to 8% as well as the parent, indicating that motility can contribute to adsorption independently of chemotaxis.

The role of motility and chemotaxis on bacterial adsorption to alfalfa roots was further examined by determining the ability of the mutants to inhibit adsorption by a derivative of the wild-type parent. When L5-30-1 (a fully motile, rifampin-resistant derivative of L5-30) was exposed to roots in the presence of high concentrations (10^6–10^7/ml) of either the parent L5-30 or behavioral mutant derivatives LP302, LP206, or LP101, its adsorption was diminished to just 1 to 2% of it normal level (A = 3.02 ± 0.32). In contrast, the addition of a heterologous, motile strain, *R. trifolii* A118, only partially (47%) inhibited adsorption of L5-30-1 to alfalfa roots. *R. trifolii* A118 was the most inhibitory of 19 different rhizobia and agrobacteria tested in a previous study (12). In other competition experiments, adsorption of the behavorial mutants was found to be almost totally (87–91%) inhibited when the competitor was a homologous motile strain, *R. meliloti* U45, but only partially (23–32%) when the competitor was *R. trifolii* A118. In these respects, the behavorial mutants behaved as other homologous (*R. meliloti*) strains (12).

In a more detailed study of the inhibition of adsorption to roots, the concentration of L5-30 and mutant derivatives as competitors was varied between 10^2 and 10^7/ml (Fig. 4). In all cases, root adsorption of the indicator strain L5-30-1 was progressively inhibited by increasing concentrations of the competitor strain at concentrations of 10^4 bacteria/ml and higher. Inhibition was essentially complete in the presence of about 10^6 competitor bacteria/ml. At intermediate concentrations, there were clear, reproducible differences in the ability of the mutants and the parent to inhibit adsorption of L5-30-1, with significantly higher concentrations of the mutants required to cause half-maximal inhibition ($P < 0.05$, t-test after arcsin transformation of values form three independent experiments).

In similar experiments, *R. trifolii* A118 was added at high concentrations in an attempt to saturate the 'nonspecific' mode of root adsorption common to both heterologous and homologous strains (12), thereby emphasizing any 'host-specific' mode of adsorption unique to the homologous interaction between *R. meliloti* and alfalfa. The ability of increasing concentrations of L5-30 and behavioral mutant derivatives to inhibit adsorption of L5-30-1 was not significantly altered by the presence of a high concentration of *R. trifolii* A118 (data not shown). In related experiments, also in the presence of high concentrations of A118, the rate of adsorption of the behavioral mutants was found to be 50 to 100 times lower than that of the parent (Fig. 5).

DISCUSSION

Several spontaneous behavioral mutants of *R. meliloti* L5-30 have been isolated and partially characterized. Ziegler *et al.* (36) recently mapped the mutations in strains LP302 (*che*-302) and

Table III. *Competitive Advantage Provided by Motility and Chemotaxis*

Alfalfa seedlings (5 d old) were inoculated with 4.6 to 5.4 × 10^4 total bacteria per plant of the individual strains or of a 1:1 mixture of parent and mutant. Nodules above the RT mark were harvested after 15 d, surface sterilized, and their contents analyzed on swarm plates to determine their phenotype.

Strain[a]	Phenotype	Percentage of Nodules Containing			Nodules Examined
		Wild type	Mutant	Mixed	
L5-30	Wild type	100	0	0	20
LP302	Che$^-$	0	100	0	19
LP206	Mot$^-$	0	100	0	20
LP101	Fla$^-$	0	100	0	23
L5-30 + LP302		76	23	1	141
L5-30 + LP206		72	26	2	155
L5-30 + LP101		87	8	5	131

[a]

Table IV. *Adsorption of Behavioral Mutants to Alfalfa Roots*

Groups of 15 5-d-old seedlings were incubated during 4 h with 10^3 to 10^4 bacteria, washed 4 times, and embedded in YEM agar supplemented with streptomycin (100 μg/ml) and cycloheximide (25 μg/ml). Microcolonies developed along the root surface were counted after a 2-d incubation at 28°C.

Strain	Phenotype	Percent of Bacteria Adsorbed to Roots (A)[a]	Relative Adsorption
L5-30	Wild type	3.0 ± 0.4	100
LP302	Che$^-$	0.51 ± 0.12	16
LP304	Che$^-$	0.57 ± 0.10	19
LP206	Mot$^-$	0.19 ± 0.05	6
LP209	Mot$^-$	0.22 ± 0.04	8
LP101	Fla$^-$	0.21 ± 0.10	7
LP107	Fla$^-$	0.16 ± 0.04	6

[a] Values are given with 95% confidence intervals.

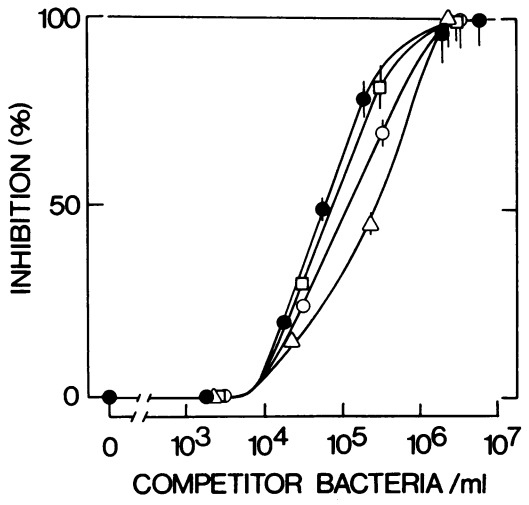

FIG. 4. Inhibition of root adsorption of *R. meliloti* L5-30-1 to roots of alfalfa cv Dawson by competitor *R. meliloti* L5-30 (●) and derivatives LP302 (○), LP206 (□), and LP101 (△). Fifteen 5-d-old seedlings were incubated during 4 h with 22.5 ml bacterial suspension containing (1.8 ± 0.1) × 10^3 of strain L5-30-1 and different concentrations of the competitor strains. After four consecutive standardized washings, seedlings were embedded in YEM agar supplemented with rifampicin (40 μg/ml) and cycloheximide (25 μg/ml) as inhibitor of plant and fungal growth, and incubated 2 d at 28°C to count microcolonies along the root surface. Vertical bars indicate 95% confidence intervals for this representative experiment. No bars are shown where the confidence interval is smaller than the symbol.

LP206 (*mot*-206) in a *fla/che* cluster near the *his* chromosomal marker of *R. meliloti*. Mutant LP101 (*fla*-101) was found to map distantly from this cluster.

The mutant isolates were all able to generate symbiotically effective nodules on alfalfa, in agreement with previous observations (3). However, the emergence of nodules generated by these mutants was significantly delayed (Fig. 1). This observation led us to investigate the role of motility and chemotaxis in the initial steps of nodule formation.

Since nodule initiation in alfalfa is developmentally restricted to the region of emerging root hairs (6; Fig 2), it is possible to distinguish by location those nodules that were initiated during the first few hours after inoculation and any nodules initiated at later times. In alfalfa, root cells located above the RT mark made at inoculation were found to remain susceptible to rhizobia for only about 8 to 12 h (6). The parent and behavioral mutants were found to increase in number less than twofold during the 12 h period following inoculation. This limited bacterial growth and the narrow time window afforded by acropetal root development make it possible to rigorously and quantitatively compare nodule initiation by the parent with its behavioral mutant derivatives, avoiding artifacts due to differential multiplication of bacteria, uncertain numbers of bacteria in contact with the host root, and feedback regulation of nodule formation.

Both L5-30 and it nonmotile mutant derivatives were able to generate considerable numbers of nodules in the initially susceptible region of the primary root (Fig. 2). The average position of the uppermost nodule on the primary root and the median position of nodules on the primary root was found to be quite similar for the parent and behavioral mutants. Because the susceptibility of alfalfa root cells to rhizobia is developmentally restricted, delayed nodule initiation would result in a shift in these positions towards younger regions of the root (7, 23, 24). We conclude that the behavioral mutants initiated nodule formation just as rapidly as the parent. Differences of about 6 to 8 h in the time required to initiate the first nodules should have been readily detectable in these assays (6). The 4-d delay in the appearance of mutant-generated nodules (Fig. 1) must therefore be due to a slower rate in the development of infections after their initiation. It will be of considerable interest to identify the specific step or steps during infection development that are enhanced by motility and chemotaxis.

The results in Table II reveal that defects in motility and chemotaxis have an appreciable effect on the average number of nodules generated in the initially susceptible region of the root

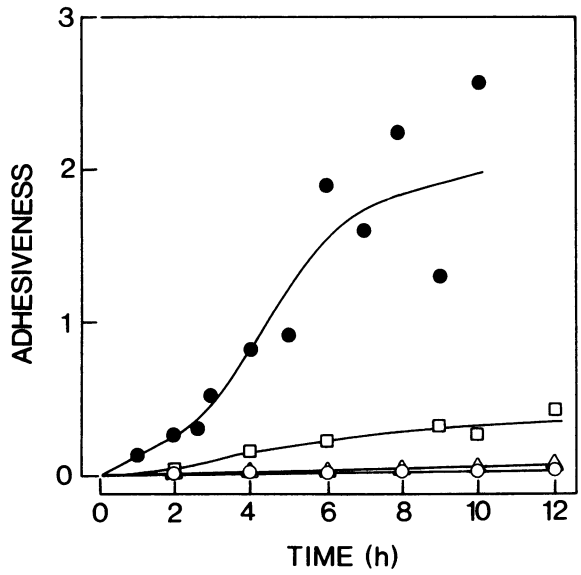

FIG. 5. Kinetics of *R. meliloti* adsorption to roots of alfalfa cv Dawson. Fifteen 5-d-old seedlings were incubated with 10^3 bacteria/ml of stain L5-30 (●), LP302 (○), LP206 (□), and LP101 (△), in the presence of 10^6 *R. trifolii* A118 cells/ml. Adsorption in the absence of heterologous strain A118 was determined at 1 and 4 h for each adsorption time course studied. The presence of A118 inhibited adsorption comparably to that seen in other experiments. Bacterial adsorption is expressed as adhesiveness (A), the percentage of bacteria in the initial suspension that adhered to the roots after washing. The 95% confidence intervals in this representative experiment were small in relation to the size of the symbols and are not shown.

above the RT mark. Superior nodulation above the RT mark by the parent cannot be explained by differential multiplication of the parent with respect to the mutants, because multiplication rates during the relevant time period in the pouches were found to be low and very comparable. In previous studies, enhanced nodulation above the RT mark by *Rhizobium* cultures was attributed to either increases in infectivity (as indicated by dose-response curves with higher slopes) or to an increased efficiency of nodule formation (as indicated by the number of bacteria required to generate half-maximal nodulation above the RT mark where dose-response curves have similar slopes) (7). Figure 3 shows that the slopes of the dose-response curves for the behavioral mutants were essentially the same as for the parent but with the curves displaced to higher dosages. Approximately 10- to 30-fold more cells of the mutant bacteria had to be added in the inoculum in order to generate as many nodules above the RT mark as the parent. It thus appears that these nonmotile and nonchemotactic mutants have approximately the same intrinsic infectivity as the parent once enough bacteria are present but are less efficient in reaching the root or getting to appropriate sites for infection.

Chemotactic movement of rhizobia in response to substances in host root exudates could play a significant role in directing the bacteria from the soil matrix to the appropriate region of the developing root. Previous studies by Soby and Bergman (34) indicated that bacterial motility and chemotaxis play an important role in the ability of *R. meliloti* to move within a soil matrix at subsaturating moisture levels. Roots are known to secrete important nutrients and chemoattractants for rhizobia (13–15, 19, 20, 28). These substances seem to be secreted primarily by cells in the zone of elongation (31, 35), and thus are coming from root cells which are or will soon become susceptible to infection by rhizobia (6; Table II). Of particular interest in this regard are recent studies from our laboratory demonstrating that luteolin, a flavonoid from alfalfa, acts as a potent chemoattractant for *R. meliloti* (G Caetano-Anollés, and WD Bauer, unpublished data). Luteolin also specifically induces expression of the common *nod* genes in this species (29, 32).

A major reason for the lower nodulation efficiency of the behavioral mutants (Fig. 3) is likely to be the lack of chemotactic movement of these bacteria to the root surface. The percentage of mutant bacteria that were able to firmly adsorb to the host root during a 4 h period of incubation was found to be 5 to 20 times lower than the percentage of parental bacteria (Table IV). These results provide clear evidence that motility and chemotaxis can contribute importantly to root adsorption. However, it is essential to recognize that the homogeneous aqueous suspensions used in these adsorption assays are quite unlike the thin discontinuous films of water and root mucigel encountered by bacteria in the rhizosphere. Assuming that the results from this *in vitro* binding studies provide a reliable indication of bacterial interactions with the roots in growth pouches, it appears that approximately half of the improved efficiency afforded by motility and chemotaxis can be attributed to a greater frequency of bacterial contact with the root. Chemotaxis was clearly more important to root contact and adsorption than motility alone (Table IV). It remains to be determined whether chemotaxis and motility also contribute importantly to nodulation efficiency by facilitating movement of bacteria on the root surface to special sites favorable for infection.

Previous studies by Bergman *et al.* (2, 22) have shown that nonmotile and nonchemotatic mutants are significantly impaired when competing for occupancy of nodules at high inoculant concentrations. The results from our competition studies are in good agreement with these earlier observations. After co-inoculation of alfalfa seedlings with suspensions containing 1:1 mixtures of the parent and one of the mutants, the number of nodules generated above the RT mark by the parent was found to be 3- to 10-fold greater than the number of nodules occupied by the mutants (Table III). The motile and chemotactic parent in these mixed strain experiments competed somewhat more effectively than one would predict from the single strain dose-response comparisons in Figure 3 at the indicated dosage of 5×10^4 bacteria/plant. Motility and chemotaxis may therefore confer some additional, as yet unidentified advantage when more than one isolate is present, perhaps involving the ability of one bacterial isolate to inhibit adsorption of another isolate to the root surface.

This possibility was examined by determining whether nonmotile or nonchemotactic mutants were able to inhibit adsorption of other rhizobia. Previous studies (12) have shown that all normal homologous (*i.e. R. meliloti*) strains tested were able to almost fully (97–99.5%) inhibit adsorption of an *R. meliloti* indicator strain to alfalfa roots, whereas heterologous strains of rhizobia were only able to partially (<60%) inhibit adsorption. From the results in Figure 4, it is clear that the Che^-, Fla^-, and Mot^- mutant derivatives of L5-30 were also able to strongly inhibit the binding of the indicator stain, although more cells of the mutants were required to achieve half-maximal inhibition. Thus, chemotaxis and motility do have some role in the competitive inhibition of bacterial adsorption to roots, but neither motility nor chemotaxis appear to be required for the observed competitive advantage of homologous strains over heterologous strains in this regard.

The low rates of adsorption for behavioral mutants seen in Figure 5 are consistent with the substantially reduced adsorption of these mutants in single time point assays (Table IV). The kinetic analysis of Figure 5 indicates that the differences in adsorption between the mutants and the parent were primarily rate differences, not differences in plateau or steady state levels of adsorption, and affirms that the choice of 4 h for single time

point experiments was a reasonable one. The presence of heterologous bacteria did not seem to influence the adsorption of the mutants relative to the parent.

We find it surprising that the rate of adsorption of the Che$^-$, Mot$^-$, and Fla$^-$ mutants was 50 to 100 times lower than the wild type under experimental conditions where the suspension of roots and bacteria was continuously agitated at 50 rpm during incubation. The inference from this result is that, relative to chemotaxis/motility, such agitation does not appreciably enhance contact between bacteria and the root surfaces, at least not the kind of contact that leads to firm adsorption.

In summary, motility and chemotaxis are clearly important factors in establishing the initial chemical and physical contact between the bacterial symbiont and the host root. Our evidence indicates that motility and chemotaxis contribute importantly to the rate and extent of contact with the root, to the efficiency of nodule initiation over and above that attributable to improved contact of bacteria with the root surface, to nodulation competitiveness, to the ability of the bacteria to inhibit the attachment of other bacteria, and to the rate of infection development and nodule emergence. It remains to be learned whether chemotaxis and motility are of comparable importance to the general saprophytic competence of these bacteria in the soil and to establishing symbiotically relevant contact between the two partners in soil environments.

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