Plant Physiol. (1992) 98, 1181–1189
0032-0889/92/98/1181/09/$01.00/0

Received for publication August 15, 1991
Accepted November 18, 1991

Growth and Movement of Spot Inoculated *Rhizobium meliloti* on the Root Surface of Alfalfa[1]

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ABSTRACT

Inoculum droplets of approximately 10 nanoliter volume and containing about 10 *Rhizobium meliloti* cells were placed onto the root surface of alfalfa seedlings in plastic growth pouches at either the root tip, the position of the smallest emergent root hairs, or at a site midway between these points. The droplets were initially confined to an area of about 0.2 square millimeter at the point of application. By 48 and 96 hours after inoculation, the inoculum bacteria and their progeny were distributed over several centimeters of the root between the initial site of deposition and the growing root tip, reaching densities of 10^3 to 10^4 bacteria per centimeter near the site of initial deposition and decreasing exponentially from that point toward the root tip. Graphite particles deposited on the root surface close to the growing tip were similarly distributed along the root length by 48 and 96 hours, suggesting that passive displacement by root cell elongation was primarily responsible for the spread of bacteria. A nonmotile mutant of *R. meliloti* colonized alfalfa roots to the same extent as the wild type and was usually distributed in the same manner, indicating that bacterial motility contributed little under these conditions to long distance spread of the bacteria. However, when applied in low numbers, *R. meliloti* mutants defective in motility or chemotaxis were considerably less efficient in initiating nodules near the point of inoculation than the wild type. This implies that motility and/or chemotaxis contribute significantly to local exploration for suitable infection sites. Almost all nodules on the primary root formed within a few millimeters of the spot-inoculation site, indicating that, under our experimental conditions, movement and multiplication of *R. meliloti* on the root surface were not sufficient to maintain an adequate population in the infectible region of the root during root growth.

In recent studies to examine the process of root colonization by introduced bacteria, seed-inoculated *Pseudomonas fluorescens* strains were able to colonize wheat roots at distances at least 7 to 9 cm from the seed under conditions in which downward percolation of water could not contribute to movement of the bacteria (18). Nonmotile mutants were found to colonize both older and younger segments of wheat roots just as well as the wild type, suggesting that bacterial motility and chemotaxis did not significantly enhance dispersal or multiplication of the inoculum strain on the root surface. Similar results were reported for colonization of soybean roots by *P. putida* (27).

Based on their observations, Howie *et al.* (18) concluded that the main mechanism for dispersal of bacteria over the surface of roots growing through soil must be passive displacement resulting from root elongation rather than active movement by the bacteria. Roots grow as a result of cell divisions in the root tip meristem followed by elongation of the newly formed cells in a region extending a few millimeters behind the meristem. Steady multiplication of bacteria adhering to the tip region should result in progeny that are initially spread apart by root cell elongation, creating low population densities in this region. Bacterial density should increase rapidly in slightly older regions, where the underlying root cells have stopped elongating, and then approach a steady level in progressively older regions of the root as the limits of nutrient availability are reached. This basic model of bacterial dispersal and root colonization is consistent with the 100-fold differences in bacterial population density observed between younger and older regions of the roots (18, 27) and with the constant population density of inoculum bacteria observed in the oldest parts of the root (27).

The studies of DeWeger *et al.* (10) provide evidence that active movement of inoculum bacteria can also contribute to dispersal and multiplication on the root surface. A wild-type strain of *P. fluorescens* was found to colonize the younger regions of potato roots about 1000-fold better than any of four independent nonmotile mutant derivatives. It is not clear why bacterial motility and/or chemotaxis had such large effects on root colonization in these experiments but not in those mentioned above. However, these results make it clear that motility and/or chemotaxis have considerable potential to modify the basic pattern of colonization resulting from passive dispersal through root cell elongation.

Further characterization of the factors that limit root colonization by introduced microorganisms are of practical as well as of fundamental interest, because rhizobacteria such as the pseudomonads examined in the above studies (10, 18, 27) can protect the roots of their host plants from invasion by pathogenic microorganisms. Such protection is commonly mediated by antibiotics or siderophores secreted into the rhizosphere (31). In such cases, the degree of protection may be simply proportional to the number of biocontrol bacteria colonizing the root.

However, for other kinds of symbiotic bacteria such as *Rhizobium*, which must infect specific root cells at specific

[1] Supported by Department of Energy grant DE-FG02–86ER13522, by grant 86-CRCR-1–2118 from U.S. Department of Agriculture Competitive Research Grants Office, by grant BSR 8819789 from the National Science Foundation, and by the Ohio Agricultural Research and Development Center.

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stages of their development, the relation between rhizosphere colonization and symbiotic benefit is more complicated. Rhizobia can normally initiate symbiotic infections leading to nodule formation in various legumes only in the region of newly emerging root hairs (2, 8, 11, 25, 26). High population densities of rhizobia in older regions of the root are thus likely to contribute nothing to nodule formation except where secondary roots emerge. Only those rhizobia in contact with the zone of young, infectible root hair cells are relevant to nodulation, and these bacterial cells or their progeny must initiate infections within the 2- to 8-h developmental time window during which the root hairs and underlying cortex are responsive.

The present study was undertaken to examine the ability of *Rhizobium* cells to move and multiply on the surface of host roots after initial contact. To accurately define the initial number and starting point of the bacteria on the root surface, alfalfa plants were inoculated by placing a very small droplet of *R. meliloti* suspension onto the root surface at a developmentally specific point in the RT[3] zone. The inoculated plants were then maintained in sterilized plastic growth pouches to minimize the effects of abrasion, desiccation, antibiosis, predation, and competition for nutrients. Nonmotile and nonchemotactic mutants of *R. meliloti* were compared against the wild type to assess the contributions of active bacterial movement. Roots were also inoculated with different numbers of *R. meliloti* cells to determine the minimum number of bacteria required to initiate a symbiotic infection. The observed patterns of movement, multiplication, and nodulation indicate that, even in the absence of competing microbes, *R. meliloti* cells do not maintain an adequate population density in the infectible zone of the host root.

MATERIALS AND METHODS

Bacterial Strains and Plant Material

Wild-type *Rhizobium meliloti* L5-30 was obtained from G. Martinez-Drets, Montevideo, Uruguay. Behavioral mutant derivatives LP101 (lacking flagella), LP206 (inactive flagella), and LP302 (nonchemotactic) have been described in previous studies (7, 32). Cultures were grown to late exponential phase (A_{500} = 0.5–0.6) in yeast extract-mannitol-gluconate liquid medium and diluted to the desired concentration (3, 4). Bacterial numbers were determined by plating onto yeast extract-mannitol-gluconate agar with a model DU plater (Spiral Systems Inc., Cincinnati, OH). Alfalfa seeds (*Medicago sativa* L. cv Vernal) were surface-sterilized (6) and germinated on inverted water agar plates at ambient temperature in the dark before transfer to pouches.

Growth and Inoculation of Alfalfa Plants

Plastic growth pouches (Northrup King Seed Co., Minneapolis, MN) were sterilized by exposure to ethylene oxide and subsequently wetted with 10 mL of sterile Jensen nitrogen-free medium (19). This volume was sufficient to moisten the entire towel to the point of saturation. Two days after imbibition, sets of five seedlings were transferred to individual pouches, inoculated, and subsequently maintained in a growth chamber under controlled environmental conditions (70–80% RH, 26°C day/24°C night, with a 16-h photoperiod and 250 μmol m^{-2} s^{-1}).

Bacteria were delivered to the root surface with a hand-drawn capillary pipet fixed to a 1-mL syringe (28). Average droplet size was determined by counting the number of bacteria delivered in individual droplets from a suspension of known bacterial concentration. Replicate droplets of the suspension were touched to the inside surfaces of sterile centrifuge tubes, vortexed with 1 mL Hoagland mineral solution (17), and then plated. It was found that droplets delivered immediately after application of pressure to the syringe were larger than subsequent droplets. With practice, it was possible to obtain an intermediate sequence of several droplets of small, uniform size for inoculation of roots in an individual pouch. To improve uniformity, it was helpful to touch each developing droplet to the root surface within a relatively constant time interval following initiation of that droplet. Between inoculations, it was often useful to touch the pipet tip to a sterile plastic Petri dish to test droplet size and/or start a new droplet. Bacterial cultures were diluted prior to inoculation so that the droplets delivered to the root surface would contain between 1 and 10^4 bacteria, depending on the experiment. Clumping of rhizobia occurred infrequently and was probably not a factor in counting or initial dispersal on the root surface.

As illustrated in Figure 1, seedlings were spot-inoculated 5 d after imbibition at either the RT, the location of the EH, or the midpoint between RT and EH. At the time of each inoculation, the locations of RT and EH were determined with the aid of a dissecting microscope at 12× magnification and marked with indelible pen on the plastic face of the pouch. The RT-to-EH distance at the time of inoculation was typically 3 to 4 mm, and this distance remained relatively constant during a 4-d growth period. Plants were discarded if the RT-to-EH distance was more than twofold greater or smaller than the 3 to 4 mm average or if the inoculum droplet was observed to spread appreciably over the root surface or onto the paper towel wick at the time of inoculation. To avoid undesired spreading of the inoculum droplets, the plastic face of the pouch was held at a distance from the root surface by placing a plastic straw next to the root and placing the pouch in a high humidity chamber for 2 h before removal of the straw and transfer to the growth chamber (Fig. 1). To prevent spread of bacteria by mass flow of water toward the bottom of the pouches, no water was added to the pouches for 96 h after inoculation. Plants maintained for analysis of nodule formation were watered 4 and 6 d after inoculation.

The method of sequential inoculation (5, 24) was used to test for feedback suppression of nodule formation in younger parts of the root by spot-inoculated bacteria. Sets of alfalfa seedlings were drip inoculated 24 h after an initial spot inoculation by applying 100 μL of the bacterial suspension evenly over the root surface, from the tip toward the base of each plant (5).

Analysis of Nodule Formation

Sets of 50 to 70 plants were analyzed to determine the distribution and average number of nodules formed in specific

[3] Abbreviations: RT, root tip; EH, smallest emergent root hair; RDU, relative distance unit.

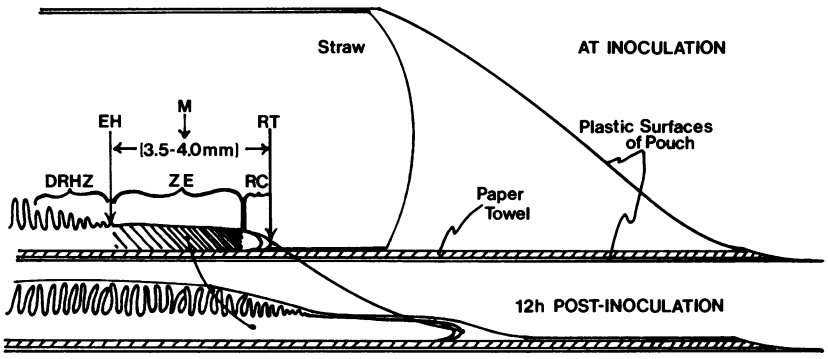

Figure 1. Spot inoculation method. An alfalfa root in a growth pouch is shown diagramatically before (upper section) and 12 h after (lower section) spot inoculation. At the time of spot inoculation and for 2 h after inoculation, the plastic face of the growth pouch was held away from the root surface with a plastic straw. Droplets containing 10 to 20 nL of a bacterial suspension were placed either at the root tip (RT), the smallest emergent root hairs (EH), or the RT-to-EH midpoint (M), as indicated by the vertical arrows. The zone of root cell elongation (ZE) extended from just back of the root cap (RC) to approximately the start of the zone of developing root hairs (DRHZ). Elongation of root cells was most rapid and extensive in the region below M, as indicated approximately by the density of shading of the root. The average distance between the RT and the EH was 3.5 to 4.0 mm (= 1 RDU). By 12 h after inoculation, the root has elongated about 5 mm. The displacement of inert particles placed on the root surface at M or RT by root elongation during this interval is indicated by curved lines. Particles placed at EH were not measurably displaced.

regions of the root. The location of each nodule on the primary root of each test plant was determined in relation to the RT mark on the pouch using a graphics tablet with digitizing pen as previously described (3). To compare results from plants with different rates of root elongation, the distance of each nodule from the RT mark is expressed in terms of RDU equal to the metric RT-to-EH distance for each individual plant (3).

Distribution of Bacteria on the Root Surface

Roots were excised in 0.5- or 1-cm-long segments, starting in each direction from the RT mark. Sets of segments from corresponding locations on 8 to 10 different roots were pooled and placed in 5-mL test tubes containing 1 mL of Hoagland solution. The tubes were placed in the cup-horn unit of a sonicator (model W370, Heat Systems Inc., Farmingdale, NY) and sonicated for 5 min at 50% power to release surface-associated bacteria (30) for plate counting. Control sets of root segments were homogenized after sonic release to determine whether release of surface-associated bacteria was complete. On average, such homogenates contained less than 10% of the cells released by prior indirect sonication.

Spread of Bacteria to the Paper Towel Wick

Sets of 10 roots were spot inoculated with approximately 10 bacteria/droplet and incubated for 48 or 96 h in growth pouches. One-cm-wide strips of the paper towel, the length of each root, were then cut with a scalpel. After removal of the roots from the pouch, the strips of towel were transferred to 2 mL of Hoagland solution. The bacteria present on the paper towel were counted by plating after release by sonication as above or after partial disruption in a blender.

Passive Spread Resulting from Root Elongation

Passive movement was assessed by measuring the displacement of graphite particles placed onto the root surface. Small graphite particles were placed at specific locations or at various intervals along the root by means of an individual hair fixed to a handle. In some experiments, the entire RT-to-EH region was uniformly covered with graphite particles using a small brush. The distance of individual particles relative to the RT mark was measured to the nearest 0.1 mm with the aid of the graphics tablet and a dissecting microscope at 25× to 50× magnification. Measurements of subsequent distribution along the root were made 48 and 96 h after marking.

Microscopic Observation of Root-Associated Rhizobia

R. meliloti cells were labeled by incubation for 45 min with 0.1% fluorescein diacetate, washed by centrifugation at 1000*g* for 10 min, resuspended in Hoagland solution, and spot-inoculated (10^3 cells/spot) onto roots at the RT-to-EH midpoint. A Zeiss I-35 microscope with a high pressure mercury lamp and a 475 nm barrier filter set was used for epifluorescence detection of bacteria on the root surface 1 to 6 h after inoculation.

RESULTS

Reproducibility of Inoculum Droplets and Spread of Bacteria on the Root Surface to the Paper Towel

Inoculum droplets either 11 ± 1.5 or 17 ± 2 nL in volume and less than 200 μm in diameter were delivered onto the root surface with one of two capillary pipets. The spread of bacteria over the root surface at the time of spot inoculation by mass flow of water from the droplet was estimated by examining the dispersion of India ink particles and congo red

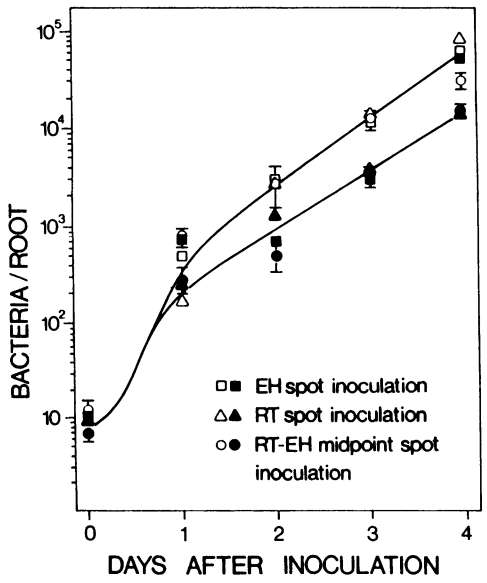

Figure 2. Bacterial multiplication on the host root surface. Alfalfa roots were spot inoculated at EH (□), RT (△), or the RT-to-EH midpoint (○) with 11-nL droplets containing either *R. meliloti* L5-30 (solid symbols) or LP101 (open symbols) at a concentration of approximately 1×10^6 cells/mL. Bacteria were recovered from the surface of the roots and counted at various times after inoculation as described in "Materials and Methods." Values are averages from three independent experiments ± SE indicated by bars.

dye. In most cases, visible spread was limited to a radius of about 0.25 mm from the site of inoculation. It was assumed that the inoculum bacteria were initially confined to a similar area, as suggested by electron microscopic observations of spot-inoculated soybean roots (28). The bacterial inoculum generally remained visible as a hemispherical droplet on the root surface for several minutes after application.

The average number of *R. meliloti* L5-30 cells that moved from the roots to the paper towel by 96 h after spot inoculation and that could be released by mild sonic vibration was about 5% of the number present on the roots. Higher percentages, from 8 to 50% of the total number of bacteria, were recovered when the paper towels were disrupted in a blender to release adhering bacteria, indicating that an appreciable percentage of the bacterial population moves from the root surface to the towel matrix and then adheres very tightly.

Bacterial Multiplication on the Host Root Surface

Sets of plants were spot inoculated at a developmentally defined location with an average of 10 *R. meliloti* cells/root. At various times after inoculation, the multiplication of these bacteria on the root surface was determined by excising intact primary roots, sonicating them to release surface-associated rhizobia, and then plating the bacteria to determine their number. As seen in Figure 2, strain L5-30 appeared to have an initial phase of rapid multiplication during which the generation time was approximately 4 h. After 24 h, the average rate of multiplication decreased and remained roughly constant, with a generation time of about 13 h. Strain LP101, a mutant of L5-30 that makes no flagella, appeared to have a slightly higher initial rate of root-associated multiplication.

Nodulation and Colonization of Roots following Spot Inoculation

The movement, multiplication, and initiation of nodules by spot inoculated rhizobia at various positions along the length of the primary root were examined in similar experiments. By 8 d after spot inoculation with 10 bacteria, nodules developed in a sharp peak at or very near the site of inoculation (Fig. 3A). No nodules formed in older regions of the primary root more than about 1 cm above the RT mark. A few nodules did develop in younger regions of the root well below the site of inoculation, but such nodules were rare, appearing only on about 2% of the plants tested. No additional nodules formed on the primary roots when nodule

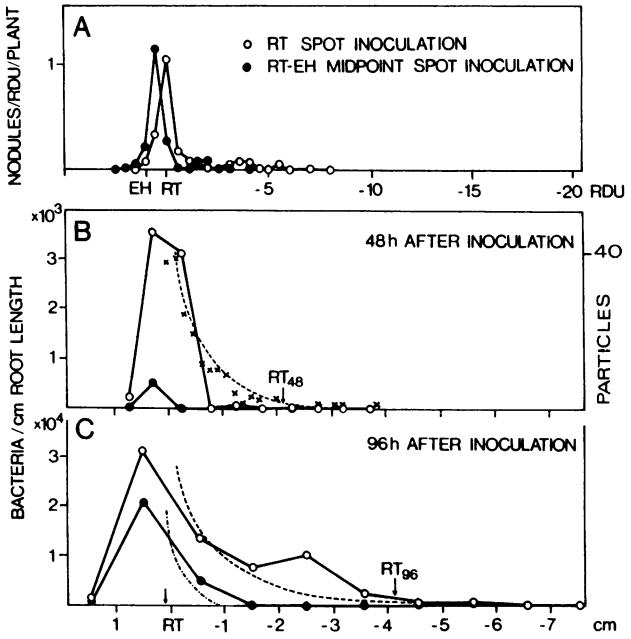

Figure 3. Nodule-distribution profiles (A) and spatial-temporal patterns of colonization (B and C) of alfalfa roots spot inoculated with *R. meliloti* L5-30. Sets of 70 seedlings were inoculated at either the RT (○) or at the RT-to-EH midpoint (●) with 11-nL droplets containing an average of 16 bacteria. The distance of each nodule on the primary root from the RT mark is expressed in RDU based on the physical RT-to-EH distance determined for the individual plant. The average RT-to-EH distance in these experiments was 3.8 ± 1.1 mm. Because the average rate of root elongation was 0.46 mm·h^{-1}, one RDU is equivalent to approximately 8 h of root growth. At 48 and 96 h after inoculation, roots were excised in 0.5- or 1-cm-long segments starting from the RT mark, and groups of 10 segments from corresponding locations were pooled and sonicated, and the released bacteria were counted as described in "Materials and Methods." Arrows indicate the average location of RT at the time of harvest. Dashed lines represent the measured distribution of graphite particles along the roots when placed uniformly over the region between RT and EH (B and C) or localized at the RT-to-EH midpoint (C). Each line is an average based on localization of several hundred particles on three individual roots.

Table I. *Nodulation of Alfalfa Spot Inoculated at Different Root Locations with R. meliloti L5-30*

Sets of 47 to 72 plants received 11- or 17-nL inoculum droplets containing an average of 10 to 16 bacteria. Nodule formation on the length of the primary root extending between the top and bottom of the pouch was scored 8 d later. Values are averages from two to three independent experiments.

Inoculation Site	Average Number of Nodules/Plant[a]	% Plants Nodulated
RT	1.38 (1.03–1.83)	52.2
RT-to-EH midpoint	0.97 (0.69–1.27)	47.1
EH	0.45 (0.28–0.64)[b]	30.3

[a] Values in parentheses are 95% confidence intervals. [b] Average differs significantly at the $P < 0.05$ level by analysis of variance test after appropriate square root transformation.

counting was delayed until 10 or 11 d after inoculation. Almost all nodules in these experiments developed singly rather than in clusters, even at the site of inoculation. The average number of nodules and the percentage of plants that developed nodules were significantly lower ($P = 0.05$) following spot inoculations at EH than spot inoculations at either the RT-to-EH midpoint or RT (Table I).

By 48 to 96 h after spot inoculation, *R. meliloti* cells were recovered from the root surfaces at considerable distances from the initial site of deposition (Fig. 3, B and C). Bacterial numbers were highest a few millimeters above the point of inoculation, reaching densities of about 3×10^3 cells/cm within 48 h after inoculation and about 3×10^4 cells/cm by 96 h after inoculation. Population densities were essentially zero at distances greater than 1 cm above the point of inoculation. They increased to maximum levels near the point of inoculation, then decreased in roughly exponential fashion in the direction of the RT. Younger regions of the root were colonized to a greater extent after inoculation at RT than after inoculation at the RT-to-EH midpoint. However, 48 h after inoculation at RT, the apical 1 cm of the root was colonized by an average of only 8 bacteria, and by 96 h colonization of the apical 1 cm diminished to an average of just 0.6 bacteria.

Microscopic observations of *R. meliloti* cells labeled by uptake of fluorescein diacetate were obscured in most places by the autofluorescence of alfalfa roots. Nonetheless, labeled bacteria were consistently seen in association with the root cap within 6 h after inoculation at the RT-to-EH midpoint. Thus, it appears that active swimming enables some of these bacteria to move about 1 or 2 mm over the surface towards the RT.

Contribution of Root Elongation to Spread of Bacteria and Inert Particles

Elongation of root cells causes younger portions of the primary root to be displaced relative to the RT mark made on the pouch at the time of inoculation (Fig. 1). The contributions of this root cell elongation to the passive spread of bacterial cells from the site of spot inoculation was estimated by determining the displacement of small graphite particles. As indicated by the dotted curves in Figure 3, B and C, inert particles applied uniformly onto the root between the EH and RT marks were displaced considerable distances (3–5 cm) by root elongation. Particle density was found to decrease in roughly exponential fashion with distance. The zone of greatest root cell elongation typically extended 0.30 to 0.45 RDU back from the RT, almost to the midpoint between RT and EH. Particles applied specifically to the RT-to-EH midpoint were displaced as much as 1 cm below the RT mark by root cell elongation (Fig. 3C). Graphite particles placed on the roots at or above EH were not measurably displaced by elongation. The colonization-distribution profiles of bacteria were similar, but not identical, to the distribution profiles of the inert particles (Fig. 3, B and C).

Effects of Inoculum Size on Nodule Distribution and Number

As shown in Figures 4 and 5, the number of bacteria delivered in the inoculum droplets clearly affected both the number and location of the nodules subsequently formed. At inoculum levels between 6 and 620 bacteria/spot, nodule formation was always highly restricted to the vicinity of the inoculation site, with more nodules at higher dosages (Fig. 4). The pattern of nodule distribution was quite different following spot inoculation with very low numbers of *R. meliloti*, averaging just 1 bacterium/spot (Fig. 4). The total number of nodules formed on the primary root was quite low. Most nodules arising from single bacterium inocula developed in younger regions of the root 5 to 10 RDU below the RT mark, with very few ever forming near the site of inoculation. The percentage of plants forming nodules in the initially suscep-

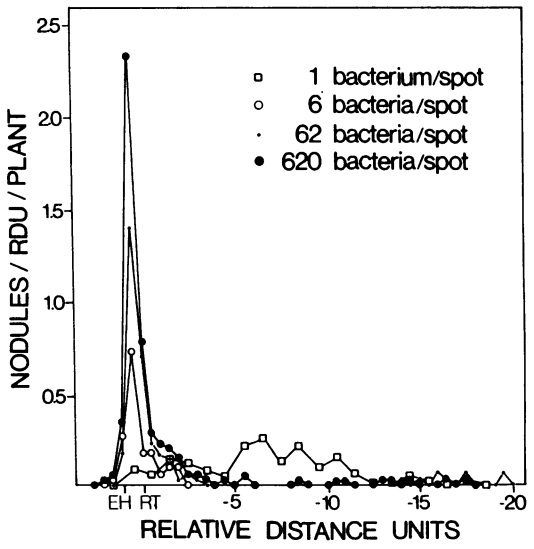

Figure 4. Nodule distribution profiles for alfalfa roots inoculated with increasing numbers of *R. meliloti* L5–30 cells. Plants were spot inoculated at the RT-to-EH midpoint with 11-nL droplets containing increasing numbers of bacteria. Nodules on the primary root were scored 8 d later as described in "Materials and Methods." Nodule locations are expressed in RDU as indicated for Figure 3. Profiles are representative of results from at least two independent experiments.

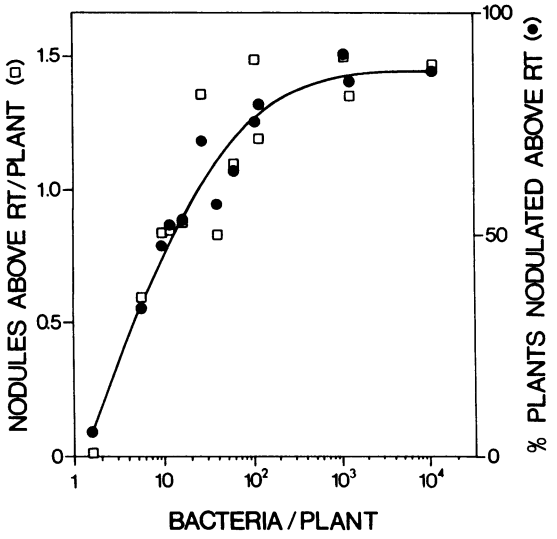

Figure 5. Effect of inoculum size on nodule number. Sets of 60 to 70 plants were spot inoculated at the RT-to-EH midpoint with 11-nL droplets containing the indicated number of *R. meliloti* L5–30 cells. Data points are average values for different sets of plants in several independent experiments.

tible region of the root, above the RT mark, increased in a linear manner with the logarithm of the number of bacteria in the inoculum droplet but reached a plateau when approximately 80% of the plants developed nodules (Fig. 5). Similar results were obtained in earlier spot-inoculation studies with *Bradyrhizobium japonicum* and soybean (28). When alfalfa seedlings from the same lot of seed were drip inoculated with similar doses of bacteria, about 85% of the roots developed nodules above the RT mark (data not shown).

Feedback Inhibition of Nodulation in Younger Regions of the Root

The sharply reduced number of nodules formed below the RT mark obtained after spot inoculations (Fig. 3) could be a result of either insufficient colonization of the infectible region of the roots or elicitation of the feedback regulatory response that governs nodule emergence in alfalfa (5). In initial controls to determine whether strain L5–30 elicits a strong suppressive response in alfalfa, roots were uniformly (*i.e.* drip) inoculated with approximately 10^5 bacteria and then drip inoculated again 24 h later at RT_2 with the same number of L5–30 cells. These experiments revealed little or no increase in nodule number in the vicinity of RT_2 attributable to the second inoculum (data not shown). Thus, strain L5–30 elicits a strong feedback suppression of nodulation, similar to that induced by *R. meliloti* strain RCR2011 (5). In subsequent experiments, roots were spot inoculated at the RT-to-EH midpoint with different numbers of L5–30 cells and then drip inoculated over the entire root surface 24 h later. As shown in Figure 6, nodulation in the vicinity of RT_2 was increasingly suppressed as higher dosages of bacteria were spot inoculated onto the root at the time of marking RT_1. Thus, at sufficiently high dosages, spot-inoculated bacteria can elicit a moderately effective feedback suppression of nodule emergence in younger regions of the root. However, in similar experiments, nodule formation near RT_2 was not measurably reduced below control levels by prior spot inoculation with either 11 or 66 bacteria/droplet (data not shown), indicating that these smaller inocula did not elicit appreciable feedback suppression of nodulation in younger parts of the root.

Role of Chemotaxis and Motility in Spread of Bacteria following Spot Inoculation

Bacterial motility and chemotaxis are traits that facilitate initial contact and adsorption of rhizobia to the host root surface and contribute significantly to competitiveness in nodule formation (1, 7, 9, 12, 21, 23). Their role in *Rhizobium* movement and multiplication on the root surface after contact was investigated by spot inoculating sets of roots at the RT-to-EH midpoint with droplets containing an average of just one cell of either L5–30 or nonmotile or nonchemotactic mutant derivatives. The overall distribution of nodules

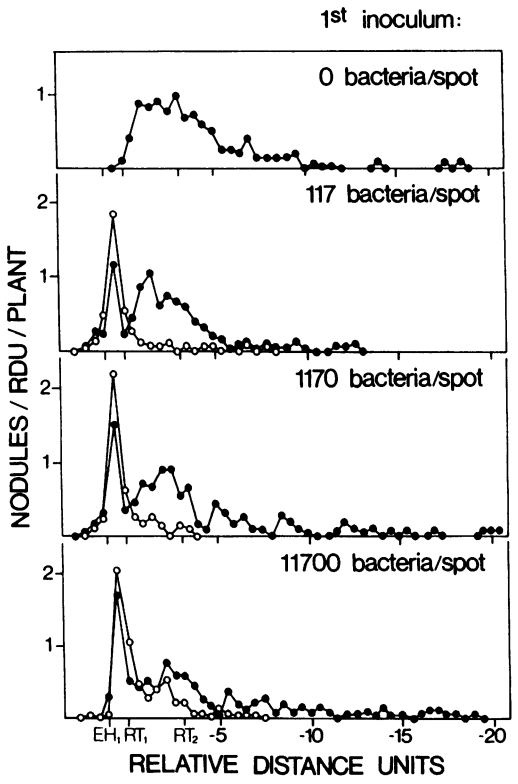

Figure 6. Nodule distribution profiles for alfalfa roots exposed to a restricted spot inoculation followed 24 h later by unrestricted drip inoculation. Sets of 50 to 60 plants were spot inoculated at the RT-to-EH midpoint with 11-nL droplets containing increasing concentrations of *R. meliloti* L5–30 at the time of marking RT_1 and then inoculated again 24 h later at the time of marking RT_2 with 100 μL of a bacterial suspension containing 4.3×10^5 *R. meliloti* L5–30 cells/mL (●) or a sham inoculum (○, Hoagland solution). Profiles are from a representative experiment. The average RT_1-to-EH_1 distance was 3.22 ± 0.41 mm, and the average RT_1-to-RT_2 distance was 9.94 ± 1.59 mm (approximately 3.0 RDU).

formed by the mutants on the primary root was not significantly different from that shown in Figure 4 for the wild type. However, as shown in Table II, the total number of nodules formed by the parent was 3 to 10 times greater ($P < 0.003$) than the number of nodules formed by the mutants. When compared at higher inoculum doses (10–35 bacteria/spot), the differences in nodulation by L5–30 and the nonmotile mutant LP101 were not as great. Root colonization profiles obtained for LP101 alternated between two patterns, sometimes like the parent (Fig. 3C) and sometimes tightly restricted to within about 1 cm of the site of inoculation (data not shown).

DISCUSSION

The dispersal and multiplication of bacteria on root surfaces after initial contact are fundamental to the dynamics of both host-microbe and microbe-microbe interactions in the rhizosphere. Unfortunately, there are serious technical difficulties to any analysis of bacterial movement and growth on root surfaces in the field. The use of spot inoculation to deliver a known number of bacteria to a particular site on the root surface provides a useful starting point for analyzing subsequent movement and multiplication, although it requires conditions that are clearly artificial.

R. meliloti was found to multiply efficiently on the root surface of alfalfa seedlings following spot inoculation. In the absence of competing microbes, the maximum growth rate of L5–30 on the root surface was reasonably close to the *in vitro* maximum (generation time, approximately 3 h). Two days after inoculation, the average rate of multiplication of the root-associated population decreased and remained roughly constant (generation time, approximately 13 h). This probably reflects the establishment of a steady-state, with rapid multiplication in newly colonized regions of the root and low multiplication in the initially colonized regions. Another *R. meliloti* strain was found to have an average generation time of about 8 h on agar-grown alfalfa roots when the seedlings were between 3 and 10 d old (20).

R. meliloti cells spread several centimeters from the original site of deposition within 48 to 96 h after spot inoculation. Based on the similarity of distribution profiles for *R. meliloti* and graphite particles (Fig. 3, B and C), it seems clear that passive displacement resulting from root cell elongation, rather than active bacterial movement, accounted for most of the spread of bacteria along the length of the root. The similarity of distribution-colonization profiles for the wild-type and nonmotile mutant LP101 is also consistent with the dominant role of passive displacement.

Nonetheless, several observations indicate that bacterial motility and chemotaxis were probably active on the root surface and significant to dispersal of the bacteria. First, it appears that there was considerable spread of the bacteria from the site of inoculation toward older regions of the root. Within 48 to 96 h after inoculation with 10 bacteria at RT or at the RT-to-EH midpoint, between 10^2 and 10^3 bacteria/cm were recovered from the root surface at distances of 5 to 20 mm above the site of inoculation (Fig. 3, B and C). Motility and chemotaxis are the most likely cause of this spread. Second, labeled bacteria spot inoculated onto the root above the zone of root elongation were microscopically observed on the root cap within a few hours after inoculation. Bacterial motility would seem to be required for this spread across the zone of elongation. Third, at low inoculum dosages, nodule formation by spot-inoculated wild-type L5–30 cells was significantly greater than nodulation by nonmotile or nonchemotactic mutants (Table II). Taken together, these observations suggest that at least some of the bacteria were able to actively move about on the root surface. Although the extent of such movement seems limited, as compared with the rate of root elongation, nonetheless it may play an important role in local exploration for sites especially favorable for infection or multiplication. In this regard, videomicroscopy has shown that *R. meliloti* is chemotactically attracted to a small number of highly localized sites in the infectible zone of alfalfa roots (13, 22). The chemoattractants released from these local sites have not been identified, but they appear to be specific to legumes and *Rhizobium* (13).

The site of initial contact between bacterium and root appears to be quite important to subsequent colonization. Initial deposition of *R. meliloti* onto the root tip consistently resulted in higher population densities along the root than inoculation at the RT-to-EH midpoint (Fig. 3, B and C). Although the physical distance between the RT and the RT-to-EH midpoint averaged only 1.5 to 2 mm, it appears that the presence of the zone of greatest root cell elongation between these two points makes them functionally quite distinct. In principle, as long as bacterial cells adhering to the RT region can multiply rapidly enough to keep up with losses due to abrasion and other factors, they can serve as a nucleus for continuous colonization of the developing root. Bacteria adhering to the root behind the zone of elongation, on the other hand, cannot colonize younger regions of the root unless they either spread actively into the zone of elongation or are carried downward by water percolation, insects, or other vehicles. The significantly higher colonization obtained by inoculation onto the RT suggests that any bacterial strain that multiplies more rapidly in the RT region, or which migrates to it more extensively, may have a significant competitive advantage in sustaining a large rhizosphere population. Fur-

Table II. *Nodulation of Alfalfa Roots Spot Inoculated with R. meliloti L5-30 or Nonmotile or Nonchemotactic Derivatives*

Sets of 46 to 58 plants were inoculated with 11-nL droplets containing an average of 0.9 to 1.5 total bacteria at the RT-to-EH midpoint. Nodulation on the primary root was scored 8 d later. Values are based on data from two independent experiments.

Strain	Genotype	Average Number of Nodules/Plant[a]	% Plants Nodulated	
			Above RT	Total
L5-30	Wild type	1.26 (0.72–1.90)b	5.2	41.4
LP101	*fla-101*	0.48 (0.15–0.89)c	0.0	18.2
LP206	*mot-206*	0.43 (0.18–0.73)c	2.0	25.2
LP302	*che-302*	0.13 (0.02–0.26)c	0.0	13.0

[a] Values in parentheses are 95% confidence intervals. Average values followed by different letters differ significantly ($P = 0.05$) by analysis of variance and Student-Newman-Keuls test after appropriate square root transformations.

ther experiments are clearly needed to test the accuracy and generality of this idea.

It is worth noting that the root cap may have a crucial role in colonization of the tip region. Root cap cells comprise a tissue that is functionally distinct and largely independent of other root tissues. During root growth, cap cells are released at a regulated rate from the cap tissue and form a patchy sheath of living cells over the root surface extending into the zone of mature root hairs (15, 29; H.E. Calvert, S. Perkins, W.D. Bauer, unpublished observations). Cap cells secrete unique chemoattractants as well as the mucilage polysaccharides that serve to lubricate the passage of the root through the soil and which may constitute a medium suitable for bacterial motility (14, 16).

Appreciable movement of wild-type *R. meliloti* from the roots to the moist paper towel beneath the roots was observed. Because the paper towel probably acts as a large sink for nutrient substances diffusing from the root, the movement of root-associated bacteria to the towel and subsequent growth in the towel near the root do not seem particularly surprising. Some of the bacteria on the towel may have moved appreciable distances through the towel and then back to the root, constituting an alternative mode of dispersal in our experiments. However, although the number of towel-associated L5–30 cells increased substantially, the number of RT-associated cells decreased from 10 to an average of less than 1 after 4 d. This indicates that any movement through the towel toward the RT did not occur fast enough or extensively enough to affect the basic exponential decay distribution pattern.

Almost all nodules generated by spot-inoculated *R. meliloti* were found to develop very near the site of inoculation. Because older root cells in the zone of mature root hairs do not respond effectively to *R. meliloti* (11), the development of nodules much above the EH mark was not expected. However, nodules rarely developed in younger regions of the root following spot inoculation, despite the fact that *R. meliloti* cells were present on the root surface in these younger regions, including the RT region where infections are initiated (Fig. 3, B and C). Feedback suppression of nodule formation cannot account for the paucity of nodules in these younger regions, because nodule formation in younger regions was minimal following spot inoculations with 11 to 66 bacteria despite the fact that these inocula failed to elicit a discernible feedback suppression response. We conclude that the number of *R. meliloti* cells in contact with the infectible region of the root during subsequent root growth was simply too low to generate an appreciable number of nodules. Approximately 8 bacteria/cm of root were associated with the tip segment 48 h after spot inoculation with 10 L5–30 cells. Only about 0.6 bacteria/cm remained after 96 h. The infectible region of the root, including the zone of elongation, is roughly 3 mm in length. Based on these values, the number of *R. meliloti* cells in contact with the infectible zone was probably between 0.2 and 3 bacteria, depending on time. The results in Figure 5 indicate that about 3% of the plants would be expected to develop a nodule in the initially infectible region if inoculated with three *R. meliloti* cells, with correspondingly lower percentages of plants nodulated when exposed to dosages of 0.2 bacteria/plant. These estimates seem consistent with the previously noted experimental value of 2% of spot-inoculated plants developing nodules in younger root regions. This correspondence argues that sparse nodulation in younger regions of spot-inoculated roots is probably a direct reflection of very low population densities of *R. meliloti* in contact with infectible host cells.

The rates of movement and multiplication of *R. meliloti* cells associated with the root do not seem fast enough to keep up with the rate of root elongation. Introduced or indigenous *R. meliloti* cells may suffer a similar fate after contacting an alfalfa root in a soil. In soils, the inability of *R. meliloti* to keep up with root growth would be compounded by losses through abrasion and inhibition by competing microbes. On the other hand, the rate of root elongation would be significantly slower in most soils than in growth pouches. Moreover, the plant and *Rhizobium* may behave differently in soil environments in ways that favor *Rhizobium* colonization. Thus, it is not appropriate to use the present results to predict success or failure of root colonization in soils. Rather, the present studies help to reveal that colonization of plant roots by bacteria is a vector sum dependent on very local rates of root elongation, bacterial multiplication, and bacterial movement.

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