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Rhizobia Are Attracted to Localized Sites on Legume Roots

MARY GULASH,† PETER AMES,‡ ROSE C. LAROSILIERE, AND KOSTIA BERGMAN*
Department of Biology, Northeastern University, Boston, Massachusetts 02115

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Clouds of *Rhizobium meliloti* were attracted to localized sites on the surface of the infectible region of alfalfa roots. This behavior, which required active motility and chemotaxis, was not species specific. Correlation between the behavior of various mutants and their competitiveness for nodulation suggests that cloud formation has a role in the infection of host legume roots by rhizobia.

The symbiotic association for nitrogen fixation in nodules of leguminous plants requires infection of the host root by appropriate rhizobia. Despite considerable work, the initiation of infection is poorly understood, and earlier, simple, surface-recognition models (7, 12) have been modified toward more complex models that emphasize the exchange of multiple signals between the host and the bacteria (5, 11).

The Fahraeus slide chamber technique (14) has been widely used to study the infection of small-seed legumes. Typically, the earliest events measured are specific attachment by rhizobia (11, 13) and root hair curling (13, 26, 27). We have observed that shortly after inoculation, motile *Rhizobium meliloti* concentrate in highly localized clouds near specific sites on the surface of alfalfa roots. In this paper, we will describe this phenomenon and the use of behavioral mutants (3) to test its significance in the infection process.

(A preliminary report of this work was presented in August 1982 at the 13th International Congress of Microbiology, Boston, Mass.)

MATERIALS AND METHODS

The media and methods used for growth and behavioral analysis of wild-type (RM2011) and mutant *R. meliloti* strains were described previously (3). NR4300, the only new mutant used in this study, was found among a collection of spontaneous mutants (R. C. Larosiliere, M.S. thesis, Northeastern University, Boston, Mass., 1984) isolated by repetitive enrichment from the center of swarm plates (3, 4). Mid-log phase, aerobic cultures grown at 30°C with shaking (200 rpm) in maltose salts medium (MSM) were washed twice in fresh MSM. Experiments were performed as long as cells of motile strains retained good motility (generally 5 to 6 h).

Seeds of alfalfa (*Medicago sativa* cv. Saranac) were purchased from Agway, Inc. For surface sterilization, seeds were washed in 95% ethanol, soaked in 0.2% $HgCl_2$ for 20 min, and then washed 5 times in sterile water (25). The seeds were then allowed to germinate on water agar plates placed upside down at 25°C. Sterility of the seeds and seedlings was routinely tested by streaking on the surface of plates of various rich bacteriological media. The surface of the seeds was always sterile, but the surface of 10 to 15% of the seedlings was contaminated with a fast-growing, motile, gram-negative rod which formed large yellow colonies overnight. A single-colony isolate of this organism was identified as *Enterobacter agglomerans* (API 1005373) by Hollis Bodman of Brigham and Women's Hospital, Boston, Mass. Presumably, it survives surface sterilization because it is under the seed coat. All seedlings were tested so that those showing this surface contamination could be eliminated from the study. Handelsman and Brill (Abstr. Annu. Meet. Am. Soc. Microbiol. 1984, K144, p. 171) have also isolated a gram-negative organism from alfalfa seedlings. They typed it as *Erwinia herbicola*, a name synonymous to *E. agglomerans* (18). We follow the *Erwinia* nomenclature since it is general usage for plant isolates.

Observations of plant roots and bacteria incubated together in standard Fahraeus slide chambers (24) were made with an Olympus BHS trinocular microscope equipped with phase optics and a video recording system (Panasonic WV1850 video camera, WV5360 monitor, and NV8320 VHS recorder). Measurements of the number of bacteria in various parts of the field and their swimming speeds were made directly off the video screen during playback. These measurements were facilitated by use of the pause mode on the video recorder and a stopwatch timer superimposed on the recordings by a time-date generator (Panasonic WJ810).

For experiments on the species specificity of the cloud formation behavior, we obtained the other strains of bacteria and cultivars of plants listed. Bacteria were as follows: *R. trifolii* 0403 (from F. Dazzo, Michigan State University), *R. leguminosarum* FH615K (from N. Amarger, Laboratoire de Microbiologie des Sols, Dijon, France), *R. japonicum* 61A76 (from W. D. Bauer, Charles F. Kettering Research Laboratory), *Escherichia coli* W3110 (from J. S. Parkinson, University of Utah), and *Bacillus subtilis* W168 (from A. L. Sonnenschein, Tufts-New England Medical Center). Plants were as follows: birdsfoot trefoil (from W. W. Currier, University of Vermont), white clover (Ladino and Louisiana nolin, from F. Dazzo), carrot (Danvers half long, purchased from Northrup King), onion (White Portugal, purchased from Lofts Pedigreed Seed, Inc.).

Details of the methods used in competition experiments were given previously (2).

RESULTS

Clouds of motile *R. meliloti* at the surface of alfalfa roots. Figure 1 is a photomicrograph of a typical cloud of *R. meliloti* at the surface of the root of a young alfalfa plant. The bacteria in the cloud are motile and appear to be swimming toward a localized source of attractant(s).

For routine observation and unbiased measurement of this behavior, we used the videotaping system described above. The clouds were small and roughly hemispherical with a radius of 50 to 100 μm, which corresponds to a volume of 0.2 to 2 nl. They occurred only in a localized region extending about 1 mm in length and starting 0.7 mm

* Corresponding author.
† Present address: Angenics, Inc., Cambridge, MA 02139.
‡ Present address: Channing Laboratory, Boston, MA 02115.

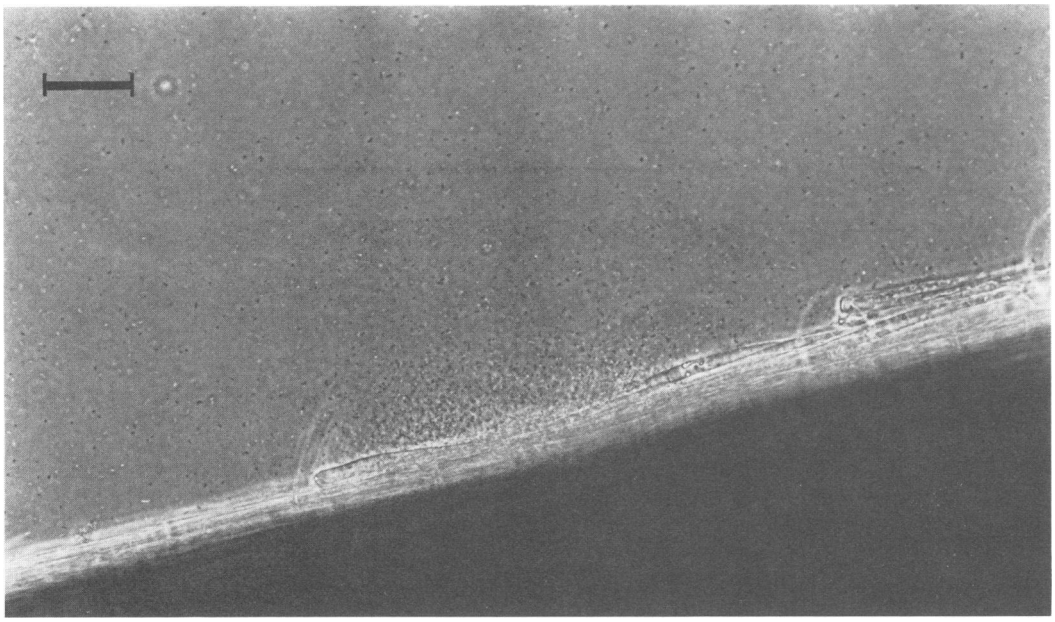

FIG. 1. A phase microscope view of a cloud of highly motile *R. melioti* at the surface of an alfalfa root. The bright line at the surface of the root is caused by light scattering. Bar, 50 μm.

up from the root tip. At least one cloud was observed near 21 of 55 plants, for an average of 0.43 ± 0.64 clouds per plant. This is undoubtedly an underestimate, since the visibility of the tiny clouds depends on their location near the comparatively enormous cylindrical surface of the root. Because of light scattering and absorption by the plant root cells themselves, only those clouds which happen to lie at or near the optical median plane of the root cylinder, as viewed in the microscope, are easily observed. Although they were probably often missed, clouds were occasionally observed over the upper surface of the root, whereas clouds under the lower surface of the root were not observed at all.

The time needed for clouds to develop depended on the inoculation procedure. Most of the observations for this study were done with standard Fahraeus slides incubated in tubes containing 30 ml of MSM. The bacteria were added to the MSM in the tube at a final concentration of 2×10^7 cells per ml, and clouds were observed 3 to 5 h later. In a new system, the slide chambers containing the plant roots were removed from the reservoir tubes and kept in a moist environment to minimize drying. The bacteria could then be inoculated directly into the 100-μl chamber. With this system, clouds were observed after 5 to 15 min but no sooner. In both methods, it was critical to change the incubating medium before adding the bacteria, because if it was not changed, no clouds developed, presumably because the gradient of attractant was too shallow to be detected above the accumulated background (19). The removal of maltose or $(NH_4)_2SO_4$ (or both) from the incubating medium had no effect on the frequency of cloud formation. However, the addition of 0.02% or more yeast extract completely eliminated cloud formation.

The clouds appeared stable in position and size with time, lasting at least 1 h, but we could not tell whether a cloud always involved the same bacteria or whether some individuals adhered to the plant and others were recruited to take their place. The average swimming speed of bacteria in a cloud (40 ± 11 μm/s) is about the same as the average swimming speed of bacteria kept in MSM.

The formation of clouds required the plant root and motile bacteria. In slide chambers containing no root or a toothpick to simulate a root, the distribution of bacteria was uniform. With two nonmotile mutants of strain RM2011, NR1000, which lacks flagella, and NR2000, which has flagella that do not rotate (3), we did not observe clouds and the distribution of bacteria was uniform.

Cloud formation is not species specific. To test the specificity of the cloud formation behavior, we used several species of bacteria and plants. The plants tested were limited to small-seed species in which the germinating root could be observed in the slide chambers. Strain RM2011 bacteria formed characteristic clouds on the roots of all the legumes tested but not on the roots of onions or carrots. Similarly, all of the *Rhizobium* strains, including representatives from several cross-inoculation groups, formed clouds on alfalfa roots. Three other motile bacterial strains were tested on alfalfa roots. *E. coli* aggregated near the plant and on the root hairs, but its motility was inhibited and it did not form clouds. *B. subtilis* accumulated in a broad area around the tip of the root but did not form characteristic localized clouds. The strain of *E. herbicola* isolated from alfalfa seedlings formed clouds of about the same size and in the same location as did the rhizobia.

Behavior of generally nonchemotactic mutant *R. meliloti* strains. Generally nonchemotactic mutations (Che^-) are those which cause the simultaneous loss of responses to all spatial gradients of attractants and repellants (4). In a recent review of work on the *che* genes in enteric bacteria, Parkinson (20) observed that point mutations in the *che* genes lead to strains with one of two different types of abnormalities in their swimming behavior; "smooth" swimmers have a greatly decreased random tumble frequency, whereas "tumbly" swimmers have a greatly increased random tumble frequency.

We characterized the behavior of two spontaneous mutants of RM2011, which we classified as Che^- because they did not respond to any attractant (including amino acids and sugars), tested on soft-agar swarm plates (3). As expected,

NR4300, which always swam smoothly in liquid culture, did not form clouds. However, quite unexpectedly, NR3000, which tumbled continuously in liquid culture, formed clouds. The bacteria in the cloud had alternating smooth-swimming and tumbling behavior similar to the wild type, except that the average smooth-swimming speed was slower (19 ± 7 μm/s). Even outside the cloud, some of the individual bacteria near the plant swam smoothly. There was always a lengthy delay of 15 to 30 min between the addition of bacteria to the slide chamber containing the alfalfa root and our first observation of smooth swimming.

Competition experiments. It has been repeatedly shown that strains vary in their competitive success at nodule production on a particular host plant (reviewed in references 17 and 24). In competition experiments, plants are inoculated with mixtures of two strains, preferably at several ratios, and then the number of nodules formed by each strain is determined. The more competitive strain will form a higher percentage of nodules than expected from its representation in the inoculum (1). Previously, we used such experiments to show that RM2011, the motile, wild-type strain, is more competitive than NR1000 and NR2000, two nonmotile mutant strains derived from it (2).

Figure 2 shows the results of competition experiments between RM2011 and the generally nonchemotactic mutant strains NR3000 and NR4300. NR3000, which tumbles continuously in pure culture but behaves more or less normally around alfalfa roots (see above), is equally competitive with RM2011. However, NR4300, which always swims smoothly, both in pure culture and around alfalfa roots, is less competitive than RM2011.

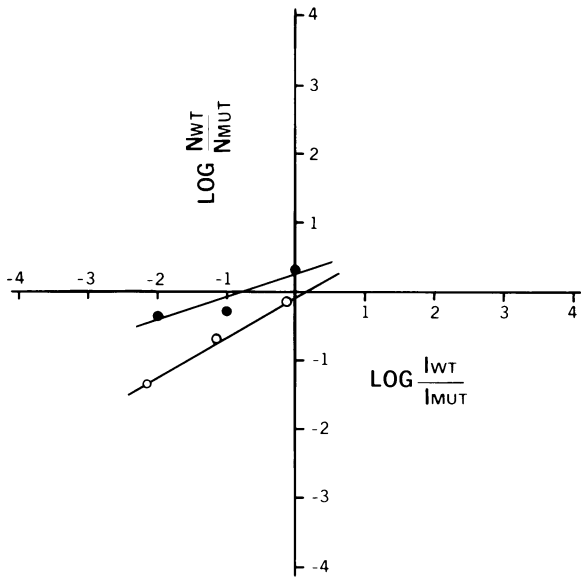

FIG. 2. Competition experiments between wild-type and mutant *R. meliloti* strains. I_{WT}/I_{MUT} is the ratio in each inoculum of the number of wild-type cells to the number of mutant cells as determined by viable cell counts. N_{WT}/N_{MUT} is the ratio of the number of nodules formed by the wild type divided by the number of nodules formed by the mutant when mixed with strains NR3000 (○) or NR4300 (●). Each point represents results from 25 to 40 nodules.

DISCUSSION

We consistently observed that rhizobia accumulate in small, localized clouds next to a very restricted region of legume roots. This region, which is above the root cap and below the region of mature root hairs, has been shown by others to be the infectible region of the alfalfa root (5, 6). Thus, legume roots may release one or more attractants that guide rhizobia to actual infection sites or at least to sites advantageous for colonization of the infectible region.

In this study, we inoculated the alfalfa plants with large numbers of bacteria (2×10^7 per ml) to reliably see the clouds. Since successful infections probably require only one individual bacterium, and at most the concerted action of 5 to 10 individual bacteria (5), the clouds may not be part of a normal infection but instead may be an exaggerated response which occurs at high culture densities under hydroponic conditions, allowing us to observe the attraction. If this is the case, the lack of species specificity observed for this response could mask a greater specificity under more physiological conditions.

In any case, the competition experiments involving mutants derived from strain RM2011 provide strong evidence for the view that chemotaxis to a localized site on the plant root has a physiological role in infection. The correlation of the unexpected cloud formation behavior and normal competitiveness of the mutant strain NR3000 is particularly suggestive. However, a definitive test will require the isolation and testing of mutants that cannot form clouds, not because of a generalized defect in motility or chemotaxis, but because they cannot detect the putative attractant.

Competition experiments in soil will be needed to test the relevance of the cloud formation response, which can only be observed under unusual hydroponic conditions, to various soil conditions.

Plant roots exude considerable amounts of organic material, including amino acids and sugars, from the general area of the root tip (22). Other researchers have shown that rhizobia are attracted to various substances in such plant root exudates (8–10, 15, 16). However, the relevance to cloud formation is unclear, since in each case the assays were performed with material from total root exudate. Since the attractant of interest, which may be a normal metabolite or a more specific signal molecule, is presumably released only from the localized sites, collection of total root exudate would dilute it by a factor of at least 10,000. Once again, when they are isolated, mutants with specific defects in chemosensors or signaling elements (21) should be useful for developing assays for the actual attractant.

What is the explanation for the dramatic change in the behavior of mutant strain NR3000 is the presence of alfalfa plants? The change occurs after a lengthy delay (15 to 30 min) and involves recovery of the ability to respond to a spatial gradient. Both of these characteristics distinguish it from the immediate smooth-swimming response observed for some tumbly *che* mutants of enteric bacteria after a large temporal change in the attractant level (20, 23). Although the change in the behavior of NR3000 does not involve all of the individuals in the culture, it involves enough so that it is not possible that we are simply observing revertants of the *che* mutation attracted into a cloud. Clearly the *che* mutation itself is conditional. After negative tests of many trivial explanations (e.g., temperature, media components, and pH), we are left with the assumption that the alfalfa root in some way modifies the environment (removes an inhibitor or adds a stimulator) so that it becomes permissive for chemotaxis by mutant strain NR3000. At this point, we cannot tell whether this change only affects the behavior of NR3000 or if it affects the behavior of the wild type as well. In preliminary experiments, we have not been able to alter the behavior of

NR3000 with plant root exudate. It may be that a higher concentration or a spatial gradient of the putative substance is required.

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