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Infection and Nodulation of Clover by Nonmotile *Rhizobium trifolii*

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Nonmotile mutants of *Rhizobium trifolii* were isolated to determine whether bacterial motility is required for the infection and nodulation of clover. The nonmotile mutants were screened for their ability to infect and nodulate clover seedlings in Fahraeus glass slide assemblies, plastic growth pouches, and vermiculite-sand-filled clay pots. In each system, the nonmotile mutants were able to infect and nodulate clover.

The infection of legume roots by the appropriate *Rhizobium* species is an initial step in establishing the nitrogen-fixing symbiotic association between *Rhizobium* and legumes (3, 4, 6). The importance of bacterial chemotaxis and motility in the infection and nodulation of legumes by rhizobia is not known. We have isolated nonmotile mutants of *Rhizobium trifolii* 2S to determine if bacterial motility is required for the infection and nodulation of clover.

Spontaneously derived nonmotile mutants of *R. trifolii* 2S were isolated by enriching for bacteria which did not migrate in semisoft agar. The center of a semisolid agar plate, mannitol-glutamate medium (2), was inoculated with bacteria. The swarm was allowed to grow for 5 days, after which time a sterile inoculating wire was inserted into the center of the swarm, and bacteria from the center of the swarm were reinoculated into the center of another sterile semisolid agar plate. This procedure, which enriched for nonmotile cells, was repeated eight times. On the eighth transfer a sterile wire was inserted into the center of the swarm, and cells from the center of this swarm were streaked to isolate colonies on solid agar. Individual colonies were screened for motility by using semisolid agar. Those bacteria which did not migrate in semisolid agar to form a swarm were designated as nonmotile. Three independently derived nonmotile mutants of *R. trifolii* 2S were isolated by this procedure. Motile revertants were isolated from each nonmotile mutant by streaking the nonmotile mutants through motility agar. The plates were incubated until a puff of motile bacteria appeared along the streak of nonmotile bacteria. Motile bacteria were isolated from the puffs.

Negatively stained preparations of bacteria were examined with the electron microscope. The motile parent and motile revertants were flagellated. All three nonmotile mutants lacked flagella.

Exponentially growing bacteria were harvested from the mannitol-glutamate broth by centrifugation at 12,000 × *g* for 10 min. The bacteria were rinsed once in sterile Fahraeus nitrogen-free mineral solution (5) and recentrifuged. The pellet was suspended in sterile mineral solution. The cell density was standardized to approximately 10^9 cells per ml and used as inocula for infectivity and nodulation assays.

The Fahraeus glass slide technique (5) was used for infectivity studies. Before microscopic examination, a sterile inoculating wire was inserted between the cover slip and microscope slide and an inoculum from the rhizosphere was stabbed into motility agar. At the time of microscopic examination, the rhizosphere was scanned for motility of the rhizobia to ascertain that reversions to motility had not occurred. The number of shepherd's crooks (7) and infection threads (5) induced by nonmotile mutants were counted 5 days after inoculation of the seedlings. Data were disregarded from any assembly which contained motile bacteria. Nodulation experiments were set up by using plastic growth pouches and vermiculite-sand filled clay pots. The infection and growth pouch nodulation data are presented in Table 1. An analysis of the variance performed on these data indicated no statistical difference ($\alpha = 0.05$) in shepherd's crooks, infection threads, or nodule numbers induced by the motile and nonmotile cells. No shepherd's crooks, infection threads, or nodules were seen on uninoculated clover seedlings grown under similar conditions.

Nodules were excised from clover roots, surface sterilized for 3 min in 0.1% $HgCl_2$, rinsed thoroughly, and crushed. Motility determinations showed that only motile cells could be

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TABLE 1. *Infectivity of motile and nonmotile R. trifolii*

R. trifolii strain	Shepherd's crooks[a]	Infection threads[a]	Nodules/ plants[b]
2S[c]	35 ± 15	8 ± 5	131:100
NM4[d]	26 ± 10	4 ± 2	102:96
NM9[d]	23 ± 9	5 ± 4	53:46
NM12[d]	22 ± 6	5 ± 4	94:86
NM4R[e]	36 ± 11	6 ± 2	90:86
NM9R[e]	30 ± 5	4 ± 3	102:103
NM12R[e]	28 ± 10	5 ± 3	89:85

[a] Four seedlings were examined for each inoculation treatment with nonmotile mutants, motile parent, and motile revertants. Two different areas, each 1 cm in length, were counted on each root. Data are expressed as the mean and standard deviation.

[b] Nodulation experiment set up in growth pouches.

[c] The motile parent.

[d] Nonmotile mutants.

[e] Motile revertants.

isolated from nodules induced by a motile inoculum. Only nonmotile cells were isolated from nodules harvested from plants inoculated with nonmotile cells when the nodules were harvested 1 week after inoculation of the seedling.

We conclude that motility of *Rhizobium* is not required for the infection of clover root hairs and formation of nodules on the roots. These findings are consistent with observations by Ames et al. (1). They have reported that nonmotile mutants of *R. meliloti* can nodulate alfalfa plants.

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This is paper number 8 in a series on host-symbiont infections.

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