JOURNAL OF BACTERIOLOGY, Mar. 1984, p. 703–707
0021-9193/84/030703-05$02.00/0

Vol. 157, No. 3

Rhizobium meliloti Competitiveness and the Alfalfa Agglutinin†

JO HANDELSMAN, RODOLFO A. UGALDE, AND WINSTON J. BRILL*

Department of Bacteriology and Center for Studies of Nitrogen Fixation, University of Wisconsin, Madison, Wisconsin 53706

Received 6 September 1983/Accepted 17 November 1983

We have isolated two types of isolates having identical colony morphologies from stock cultures of two different *Rhizobium meliloti* strains. One isolate was agglutinated at a high-dilution titer (HA, highly agglutinable) of the alfalfa agglutinin and was sensitive to phage F20, and the other was agglutinated at a lower agglutinin titer (LA) and was sensitive to phage 16B. All LA isolates from the original slant produced nodules on alfalfa earlier than did HA strains from the original slant. When these HA and LA strains were mixed and used as the inoculum in both vermiculite and field soil in the laboratory, LA strains were always the predominant strains recovered from the nodules. LA strains were obtained from HA cells by selection for resistance to phage F20, and HA strains were obtained from LA cells by selection for resistance to phage 16B. All of the strains with the HA phenotype that were derived from LA strains by phage selection had the nodulation properties of the HA strains from the original slant. Two classes of strains with the LA phenotype were obtained from HA cells by phage selection. One was identical to the original LA strains from the slant, and the other had the nodulation properties of the HA strains. Thus, we have shown that some cell surface properties change the nodulation abilities of *R. meliloti* strains and, furthermore, that specific phages can be used to enrich for more competitive rhizobia.

Symbiotic nitrogen fixation is an economically important process since the nitrogen that is supplied by rhizobia to legumes replaces costly industrially fixed nitrogen. In the laboratory, superior *Rhizobium* strains have been identified (12), but most of these strains are not agriculturally useful because less effective indigenous strains in the soil outcompete the added inoculants (3, 5, 8–10, 13, 19, 20, 22). Knowledge of the process of infection and the bases of competitiveness potentially could be applied to make desirable *Rhizobium* strains more competitive under field conditions. Studies of early events in the formation of a nitrogen-fixing root nodule have focused on seed lectins and agglutinins that seem to bind specifically to the *Rhizobium* species that normally nodulates the plant. Examples are a soybean lectin that binds to some *R. japonicum* strains (2), a clover lectin that binds to *R. trifolii* (7), and an alfalfa agglutinin that binds to *R. meliloti* (16). The reaction between the plant lectin or agglutinin and the specific *Rhizobium* species may not be essential for nodulation, however, since certain varieties of soybean that seem to lack the seed lectin have normal nodulating properties (17).

The following results demonstrate that the surfaces of *R. meliloti* cells can be altered to interact less well with the alfalfa agglutinin, yielding strains that are more competitive in laboratory competition experiments in both vermiculite and soil.

MATERIALS AND METHODS

Bacterial and phage cultures. *R. meliloti* 102F51 and 102F28 were obtained from Nitragin Co., Milwaukee, Wis. Cells were grown in a yeast extract-mannitol medium (AMA) for 3 days with good aeration (11). Cultures were streaked on AMA plates containing 15 g of agar per liter, and isolated colonies were picked and recultured for agglutination and phage testing. *R. meliloti* phage F20 was a gift from Ethan Signer. Phages 16B nd 3A were isolated from Wisconsin soils. Strains and their derivatives are shown in Fig. 1.

Nodulation of alfalfa. Alfalfa seeds (*Medicago sativa* L.), cultivar Vernal, were obtained from Olds Seed Co., Madison, Wis., and other cultivars were obtained from Stanley Duke, Department of Agronomy, University of Wisconsin. Seeds were surface sterilized in concentrated sulfuric acid for 15 min. The seeds were washed and shaken at room temperature for 2 days in sterile distilled water. The seed coats that remained attached to the seedlings were removed with sterile forceps, and the seedlings were placed on sterile germination plates. For the time course nodulation experiments, seedlings were grown in glass vials as described previously (11). For competition experiments, the seedlings were grown in porcelain pots filled with vermiculite or in plastic beakers containing soil from Madison, Wis. Initially, 5 ml of plant nutrient solution (11) was added to each plant, and distilled water was added as needed.

Recovery of nodule bacteria. Three-week-old plants were washed, and the roots were sterilized in 95% ethanol for 30 s and in 0.1% acidified $HgCl_2$ for 1 min. The plants were washed in sterile distilled water, and the nodules were removed with sterile forceps. All of the nodules from one plant were crushed with a sterile glass rod in 3 drops of AMA and streaked on AMA plates.

Preparation of the phage. Equal volumes of a phage lysate and an exponentially growing culture were incubated together at room temperature for 15 min, and then 0.1 ml of the mixture was added to 3 ml of AMA containing 0.4% agar at 45°C. The agar was poured onto an AMA plate, allowed to solidify, and then incubated overnight at 30°C. The soft agar was then scraped off and suspended in 3 ml of AMA. A few drops of chloroform were added to release the phage remaining in unlysed cells, and the agar was removed by centrifugation for 10 min at 13,000 × *g*. The supernatant solution containing the phage was removed and centrifuged again, and the resulting supernatant solution was stored at 4°C with a few drops of chloroform. Phage sensitivity was tested by picking isolated colonies and patching them on an AMA

* Corresponding author.

† Dedicated to the memory of Meng-yun Liu from the Nanjing Agricultural College, People's Republic of China.

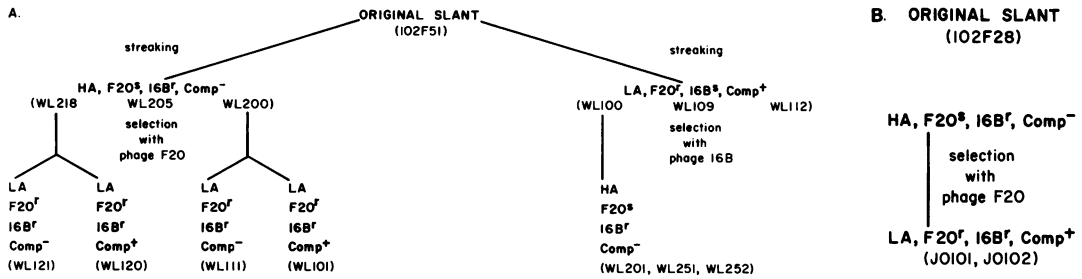

FIG. 1. Derivation of strains from *R. meliloti* 102F51 (A) and 102F28 (B). Strain numbers are in parentheses. HA strains have agglutination titers of greater than 1/160; LA strains have agglutination titers of less than 1/20. $F20^s$, Sensitive to phage F20; $F20^r$, resistant to phage F20; $16B^s$, sensitive to phage 16B; $16B^r$, resistant to phage 16B; $Comp^+$, competitive, $Comp^-$, less competitive.

plate spread with 10^8 phage. After 12 to 24 h at 30°C, the patches were examined for sensitivity.

Agglutinin-mediated agglutination assay. The alfalfa agglutinin was prepared as previously described (16). Bacterial cells were centrifuged at 10,000 × *g* for 10 min, suspended in phosphate-buffered saline, and centrifuged again. The pellet was suspended in 0.1 M sodium acetate at pH 4.0, and the cell density was adjusted to an absorbance of 2 at 660 nm. Equal volumes of the cell suspension and serial twofold dilutions of a fivefold dilution of the stock of the alfalfa agglutinin were mixed and examined for clumping after 2 h.

RESULTS

Identification of LA and HA phenotypes. In our studies of bacterial interaction with the alfalfa agglutinin, we found that single colonies of a streak from a slant of *R. meliloti* 102F51 behaved differently from each other in their ability to be agglutinated by the alfalfa seed agglutinin. The colonies could not be distinguished from each other by colony type, growth rate on a variety of media, or protein pattern by polyacrylamide gel electrophoresis (18). Of 40 colonies tested from a single slant, 25 had a low agglutinability (LA) since they were agglutinated with a titer (1/10) of agglutinin that much lower than the titer (1/320) observed with a culture from the original slant (Fig. 1A). The 15 remaining colonies tested from the streak had a titer of 1/320 (highly agglutinable, HA). When an HA or LA culture derived from a single colony was streaked again to obtain single colonies, the progeny (20 tested from each culture) had the same agglutinability as the parent cell.

The mutants WL113 and WL131 that were described previously (16) as nonnodulating and nonagglutinating mutants (previously tested at only one agglutinin concentration) were later demonstrated to be agglutinated at a low titer (1/10). In the original agglutination assay, the cell suspension had an absorbance of 12 at 660 nm, whereas in the presently discussed assay we used a cell suspension with an absorbance of 2. Thus, we can observe agglutination of much less agglutinable cells. Since all of the other LA strains discussed in this paper induce nodules on alfalfa plants, the lowered agglutinability is not responsible for the nonnodulating phenotype of mutants WL113 and WL131. Highly agglutinable variants of WL113 and WL131 have been obtained, and they do not induce nodules on alfalfa (data not shown). The cause for the nonnodulating phenotype of WL113 and WL131 has not been identified. Since most of the clones from the original culture were of the LA type, the original nonnodulating mutants WL113 and WL131 (16) were probably derived from an LA parent cell.

Phage sensitivities and selections. Since cell agglutination involves surface structures, it seemed likely that attachment of certain phage would be different in the HA and LA cells. Phage F20 lysed HA but not LA clones on AMA agar plates. Phage 16B lysed only LA clones (Fig. 1A). Another phage, 3A, lysed both HA and LA strains.

Phages F20 and 16B provided a method for directly selecting LA cells from HA cells and HA cells from LA cells (Fig. 1A). Cultures derived from single colonies of each cell type were mixed with the appropriate phage and spread on AMA plates. After 2 days at 30°C, colonies appeared at a frequency of between 2.6×10^{-5} and 6.3×10^{-6} for either phage, and these colonies were picked, purified, and checked for resistance to the phages. Half of the clones from the selection with phage F20 had low agglutinability (titers of less than 1/20), and 90% of the clones from the selection with phage 16B had high agglutinability (titers of greater than 1/160 with the alfalfa agglutinin). Presumably, these mutants had covered, uncovered, or altered the phage and agglutinin receptor, whereas the other clones were resistant due to defects in different steps in phage development. Most of these other strains had obvious defects such as altered growth rates and were not further studied since the defects could not be defined as they could be in the strains with altered agglutinability.

The phage F20-sensitive strains with the HA phenotype that were derived from LA cells by selection with phage 16B were used for further selection. When phage F20-resistant strains arose from these mutants, they were all HA. Therefore, the selected strains with the HA phenotype were different from the original HA strains since the derived strains did not give rise to LA mutants.

Another wild-type strain, *R. meliloti* 102F28, which is distinguishable by polyacrylamide gel electrophoresis (18) from strain 102F51, demonstrated HA agglutination activity and was sensitive to phage F20. In an HA culture that was grown from a single colony and plated with phage F20, the frequency of appearance of phage-resistant clones was 10^{-5} cells (Fig. 1B). All of the 20 resistant clones that were tested had low agglutinability (Fig. 1B).

Time of nodulation by HA and LA strains. Since *Rhizobium* surface structures probably play an important role in nodulation, we compared the times of appearance of nodules induced by the HA and LA strains. Based on the lectin hypothesis, our prediction might be that the HA strains that recognize the agglutinin bettter would nodulate more efficiently. This is not what we fould. Alfalfa seedlings were inoculated with either an HA or an LA culture, and nodules were counted on the plants. The mean number of nodules

per plant observed for up to 11 days is shown in Fig. 2. This graph was developed by using strains WL100 and WL200, but it is representative of many other HA and LA strain combinations tested. The results did not differ when 10^2 cells per plant were used.

To make the testing of many strains possible, a 6-day harvest assay was used. In this assay the nodules on 50 plants were counted 6 days after inoculation with either an HA or an LA strain. The HA strains (WL200, WL205, and WL218) from the original slant of 102F51 all induced significantly fewer nodules by 6 days than had all of the LA strains (WL100, WL109, and WL112) from the original slant of 102F51. All of the differences were significant at the 99% confidence level. All of the mutants with an HA phenotype (WL201, WL251, and WL252) that were selected with phage 16B from LA parents induced fewer nodules on plants by 6 days after inoculation than did their LA parent WL100. Both of the mutants with an LA phenotype (JO101 and JO102) that were derived from the other *R. meliloti* wild-type strain 102F28 induced more nodules on alfalfa plants by 6 days after inoculation than did their parent.

Two nodulation phenotypes were observed among the mutants with the LA phenotype that were selected by resistance to phage F20 from 102F51 HA parents. The first class (WL101 and WL120), which represented about half of the selected strains, induced more nodules than did their HA parents at 6 days after inoculation. The second class (WL111 and WL121) did not produce a significantly greater number of nodules at 6 days after inoculation. All of the mutants presented in this section were derived from independent selections and are thus not siblings.

These results, taken together, show that all of the original HA strains and the mutant strains with the HA phenotype nodulate more slowly than the LA strains from the original slant. All of the original LA strains and some of the mutants with the LA phenotype nodulate more rapidly than their HA counterparts. The phage F20-resistant mutants with the LA phenotype that nodulate at the same rate as their HA parents must have acquired the LA phenotype by a mechanism different from that of the other mutants with the LA phenotype that nodulate more rapidly. It would appear that the LA phenotype is required but not sufficient for the rapid nodulation phenotype.

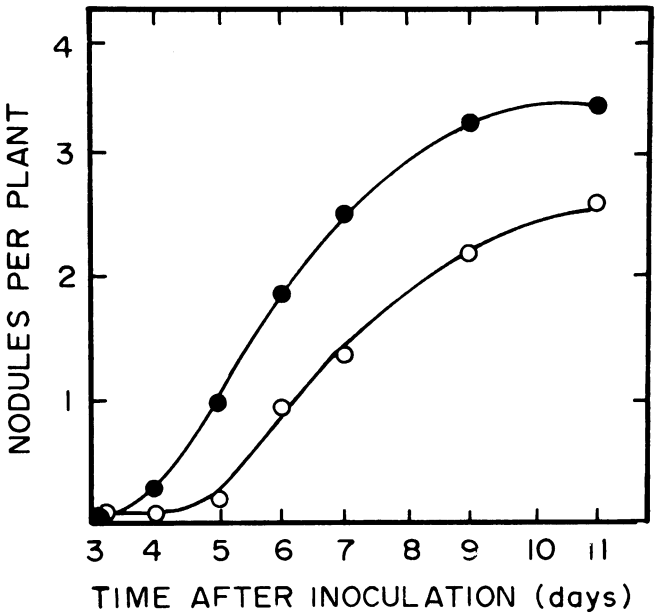

FIG. 2. Nodulation of alfalfa by highly agglutinable strain WL200 (○) and less agglutinable strain WL100 (●). Curves differed significantly at all points at the 95% confidence level in the Student's *t* test.

TABLE 1. Percentage of LA isolates recovered from nodules of plants inoculated with pure and mixed cultures

Inoculum		Host variety	%[a] of LA isolates from nodules
HA	LA		
WL200		Vernal	0
	WL100	Vernal	100
WL200	WL100	Vernal	79[b]
WL218	WL100	Vernal	91[b]
WL205	WL100	Vernal	85[b]
WL200	WL109	Vernal	75[b]
WL200	WL112	Vernal	87[b]
WL201	WL100	Vernal	90[b]
WL200	WL101	Vernal	87[b]
WL200	WL111	Vernal	49
WL218	WL120	Vernal	73[b]
WL218	WL121	Vernal	54
WL251	WL100	Vernal	80[b]
WL252	WL100	Vernal	86[b]
102F28		Vernal	0
	JO101	Vernal	100
102F28	JO101	Vernal	97[b]
102F28	JO102	Vernal	80[b]
WL200	WL100	Saranac	75[b]
WL200	WL100	Hairy Peruvian	83[b]
WL200	WL100	Ranger	85[b]
WL200	WL100	NC8376	80[b]

[a] Numbers represent the mean of the results from 20 plants. Fifty colonies were tested from each plant.

[b] Values differed significantly from 50% at the 99% confidence level as shown by the Student's *t* test. See the text for details of isolation and identification of infecting strains.

Competitiveness of HA and LA strains. Since most of the LA strains nodulated plants earlier, they might have a competitive advantage. Alfalfa seedlings were inoculated with a mixture of equal amounts (1×10^9 cells each) of HA and LA cultures or with 2×10^9 cells of either of the cultures alone. Isolates from nodules of plants inoculated with a single HA or LA strain contained only the phenotype of the inoculum, as demonstrated by phage sensitivity and agglutination titer (Table 1). Plants inoculated with any of 12 different HA-LA mixtures had 73 to 97% LA bacteria in their nodules. The LA strain also predominated when WL100 and WL200 were added to different alfalfa varieties (Table 1). LA strains JO101 and JO102 comprise 97 and 80%, respectively, of the bacteria recovered from nodules of plants inoculated with either of these strains plus their parent strain, *R. meliloti* 102F28. WL111 and WL121, which are mutants with the LA phenotype, are not more competitive than their HA parents. These are the same strains that did not nodulate earlier than their HA parents. Thus, it seems that there is a perfect correlation among these strains between the ability to induce nodules earlier and better competitiveness. Furthermore, it seems that the LA phenotype is required for both of these abilities, since all of the strains with the HA phenotype nodulated later and were less competitive and most of the strains with the LA phenotype nodulated earlier and were more competitive. The exceptions, WL111 and

WL121, show that the LA phenotype, although probably required, is not sufficient for the superior competitiveness. The difference between these two strains and the more competitive LA strains is being investigated.

The LA strain WL100 was more competitive not only in vermiculite but also in soil in the plant growth chamber. Strains WL100 and WL200 were mixed and used for the inoculum for plants that were grown in nonsterile field soil in beakers. WL100 represented 77% of the isolates from the nodules of these plants. This was significantly different from 50% at the 99% confidence level. When half as many cells were used as the inoculum and either WL100 or WL200 was inoculated by itself, an indigenous strain (EK500) was isolated from the nodules. The LA strain was more competitive than the HA strain against EK500. WL100 represented 96% of the nodule isolates from plants that had been inoculated with WL100, but WL200 represented only 80% of the nodule isolates from plants that had been inoculated with the same amount of WL200. The difference was statistically significant at the 99% confidence level in a Student's *t* test.

DISCUSSION

A hypothesis for the recognition of rhizobia by their respective host plants predicts that a plant lectin on the root surface specifically binds to a surface polysaccharide on the infecting *Rhizobium* strain (6). The data that are presented here show that *Rhizobium* strains with less ability to be agglutinated by the alfalfa agglutinin actually are better nodulators than those that are highly agglutinable. Since the mutants that were originally described as nonagglutinating were shown here to be agglutinated at a low titer of agglutinin, it is not known whether any agglutination activity is required for nodulation or whether mutants that are not agglutinated at all would be even more competitive than the LA mutants. It will be important to find out why some of the LA mutants that were obtained by phage selection are not more competitive than their HA parents. It seems that a specific type of LA cell is more competitive than other types, but we have not identified what differentiates this cell type.

The high frequency of conversion between HA and LA might be indicative of the radically different ecological niches used by rhizobia: the soil and the plant infection thread (15). LA strains have a selective advantage during nodulation, whereas HA strains may be at an advantage during asymbiotic growth. It should be interesting to determine whether most *Rhizobium* strains isolated from field-grown plant nodules have the LA phenotype. Subculturing the LA strains on laboratory media may be the selection for HA strains. A single colony from a streak of 10 different wild-type *R. meliloti* strains from stock cultures was tested for agglutinability. Five strains (2011, RM43, RM103, 102F51, and 102F28) were HA, and the rest (RM41, 104B6, 104A14, 104A12, and 102F65) were LA.

If the agglutinin is responsible for attachment of *R. meliloti* to alfalfa roots, the tight binding between *R. meliloti* and the agglutinin on the alfalfa root surface may delay nodulation and decrease the efficiency of nodulation. This is consistent with a hypothesis that suggests that agglutinins protect plants from infection by bacteria (4). This hypothesis has been developed based on plant-pathogen interactions in which avirulent bacteria are agglutinated by plant agglutinins and virulent bacteria are not agglutinated (21). It is conceivable that the alfalfa-*R. meliloti* symbiosis evolved from a pathogenic relationship since rhizobia that escape from the infection thread into the root-hair cell kill the plant cell (14). If the system were once a pathogenic one, then the plant has evolved to the position of greater advantage since it receives the fixed nitrogen from the bacteria, but the bacteria eventually decay and die in the nodule (15).

It is possible that the binding of *R. meliloti* by the alfalfa agglutinin plays no role in specificity or in attachment of the bacteria to the root. The agglutination titer may be due to a surface characteristic that helps the bacteria to survive or multiply in the alfalfa rhizosphere. Perhaps coevolution of *R. meliloti* and alfalfa has selected for chemotaxis to, or degradation of, the alfalfa agglutinin by *R. meliloti*. Therefore, the agglutinin-binding reaction may not be necessary for nodulation but may play a more subtle role in the ecology of *R. meliloti*.

In summary, we have demonstrated that (i) *R. meliloti* strains with altered ability to recognize the alfalfa agglutinin have different phage sensitivities and (ii) most of the strains that recognize the agglutinin less well induce earlier nodule formation and are more competitive in nodulation in both vermiculite and soil than are those that are agglutinated more strongly by the agglutinin. We conclude that one type of surface change that results in the LA phenotype is responsible for the earlier nodule formation and competitiveness of these strains. These surface properties may be important to consider for optimizing *Rhizobium* strains for commercial inoculant production.

ACKNOWLEDGMENTS

This research was supported by the College of Agricultural and Life Sciences, University of Wisconsin, by U.S. Department of Agriculture Science and Education grant 82-CRCR-1-1030, by National Science Foundation grant PCM-8203759, and by the McKnight Foundation. J.H. received support from Cellular and Molecular Biology Training Grant 5T 32GM07215 from the National Institutes of Health. R.A.U. was supported by the Consejo Nacional de Investigaciones Científicas y Técnicas, Republica Argentina.

We are grateful to Walter T. Leps for isolating phages 16B and 3A and to Susan J. Brooks and Alan Paau for critical comments about this work. We acknowledge the expert technical assistance of John P. Meyers, Douglas Mielke, Robert E. Vlach, Ricky Waniger, and Evenlyn Wendt-Pienkowski. We thank Irene Slater for typing the manuscript.

LITERATURE CITED

1. **Abel, G. H., and L. W. Erdman.** 1964. Response of Lee soybeans to different strains of *Rhizobium japonicum*. Agron. J. **56**:423–424.
2. **Bohlool, B. B., and E. L. Schmidt.** 1974. Lectins: possible basis for specificity in *Rhizobium*-legume root nodule symbiosis. Science **185**:269–271.
3. **Boonkerd, N., D. F. Weber, and D. F. Bezdicek.** 1978. Influence of *Rhizobium japonicum* strains and inoculation methods on soybeans grown in rhizobia-populated soil. Agron. J. **70**:547–549.
4. **Bradshaw-Rouse, J. J., M. H. Whatley, D. L. Coplin, A. Woods, L. Sequeira, and A. Kelman.** 1981. Agglutination of *Erwinia stewartii* strains with a corn agglutinin: correlation with extracellular polysaccharide production and pathogenicity. Appl. Environ. Microbiol. **42**:344–350.
5. **Caldwell, B. E., and G. Vest.** 1970. Effects of *Rhizobium japonicum* strains on soybean yields. Crop Sci. **10**:19–21.
6. **Dazzo, F. B., and D. H. Hubbell.** 1975. Antigenic differences between infective and noninfective strains of *Rhizobium trifolii*. Appl. Microbiol. **30**:172–177.
7. **Dazzo, F. B., W. E. Yanke, and W. J. Brill.** 1978. Trifoliin: a *Rhizobium* recognition protein from white clover. Biochim. Biophys. Acta **539**:276–286.

8. **Gibson, A. H., R. A. Date, J. A. Ireland, and J. Brockwell.** 1976. A comparison of competitiveness and persistence amongst five strains of *Rhizobium trifolii*. Soil Biol. Biochem. **8**:395–401.
9. **Ham, G. E.** 1976. Competition among strains of rhizobia, p. 144–150. *In* L. D. Hill (ed.), World soybean research. Interstate, Danville, Ill.
10. **Johnson, H. W., V. M. Means, and C. R. Weber.** 1965. Competition for nodule sites between strains of *Rhizobium japonicum* applied as inoculum and strains in the soil. Agron. J. **57**:179–185.
11. **Leps, W. T., W. J. Brill, and E. T. Bingham.** 1980. Effect of alfalfa ploidy on nitrogen fixation. Crop Sci. **20**:427–430.
12. **Maier, R. J., and W. J. Brill.** 1978. Mutant strains of *Rhizobium japonicum* with increased ability to fix nitrogen for soybean. Science **201**:448–450.
13. **Noel, K. D., and W. J. Brill.** 1980. Diversity and dynamics of indigenous *Rhizobium japonicum* populations. Appl. Environ. Microbiol. **40**:931–938.
14. **Nutman, P. S., C. C. Doncaster, and P. J. Dart.** 1973. Infection of clover by root-nodule bacteria. The British Film Institute, 42/43 Lower Marsh, London S.E.1.
15. **Paau, A. S., C. B. Bloch, and W. J. Brill** 1980. Developmental fate of *Rhizobium meliloti* bacteroids in alfalfa nodules. J. Bacteriol. **143**:1480–1490.
16. **Paau, A. S., W. T. Leps, and W. J. Brill.** 1981. Agglutinin from alfalfa necessary for binding and nodulation by *Rhizobium meliloti*. Science **213**:1513–1514.
17. **Pull, S., S. Pueppke, T. Hymowitz, and J. Orf.** 1978. Soybean lines lacking the 120,000-dalton seed lectin. Science **200**:1277–1279.
18. **Roberts, G. P., W. T. Leps, L. E. Silver, and W. J. Brill.** 1980. Use of two-dimensional polyacrylamide gel electrophoresis to identify and classify *Rhizobium* strains. Appl. Environ. Microbiol. **39**:414–422.
19. **Roughley, R. J., W. M. Blowes, and D. F. Herridge.** 1976. Nodulation of *Trifolium subterraneum* by introduced rhizobia in competition with naturalized strains. Soil Biol. Biochem. **8**:403–407.
20. **Semu, E. D., J. Hume, and C. T. Corke.** 1979. Influence of soybean inoculation and nitrogen levels on populations and serogroups of *Rhizobium japonicum* in Ontario. Can. J. Microbiol. **25**:739–745.
21. **Sequeira, L., and T. L. Graham.** 1977. Agglutination of avirulent strains of *Pseudomonas solanacearum* by potato lectin. Physiol. Plant Pathol. **11**:43–54.
22. **Weaver, R. W., and L. R. Frederick.** 1973. Effect of inoculum rate on competitive nodulation of *Glycine max* L. Merrill. I. Greenhouse studies. Agron. J. **66**:233–236.