APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Sept. 1991, p. 2635–2639
0099-2240/91/092635-05$02.00/0

Vol. 57, No. 9

Chemotaxis of *Bradyrhizobium japonicum* to Soybean Exudates

W. MARK BARBOUR,† DENNIS R. HATTERMANN,‡ AND GARY STACEY*

Center for Legume Research, Department of Microbiology and Graduate Program in Ecology, The University of Tennessee, Knoxville, Tennessee 37996-0845

Received 25 March 1991/Accepted 20 June 1991

The chemotactic response of *Bradyrhizobium japonicum* toward soybean seed and root exudates was examined. Assays using various isoflavones and fractionated exudate indicated that isoflavones are not the principal attractants in exudates. Likewise, induction of *nod* genes with isoflavones or seed exudate before assay did not enhance chemotaxis. Screening of numerous compounds revealed that only dicarboxylic acids and the amino acids glutamate and aspartate were strong attractants. The presence of glutamate, aspartate, and dicarboxylic acids in appreciable concentrations in soybean seed and root exudates indicates that these compounds likely represent natural chemoattractants for *B. japonicum*.

The gram-negative soil bacterium *Bradyrhizobium japonicum* forms a symbiotic relationship with soybean by way of infection of a defined region of the elongating root (8, 9). This process (i.e., nodulation) results from a series of complex events which are not fully understood. Evidence from *Rhizobium* spp. suggests that motility and chemotaxis may be involved in the early stages of this interaction by allowing the bacterium to spread through soil (26), effectively locate infection sites (16), nodulate the plant more efficiently (10), and more effectively compete for nodule formation (3, 21). Likewise, motility has been reported to be a factor in competition for nodulation between *B. japonicum* strains; nonmotile mutants were shown to form only 20% of the nodules on plants inoculated with equal numbers of motile and nonmotile bacteria (17).

Flavonoid molecules exuded by seeds and roots of leguminous host plants are required for transcriptional induction of the nodulation genes in (*Brady*)*rhizobium* species (reviewed in reference 20). The isoflavones, genistein and daidzein, are found in soybean seed and root exudates (24, 25) and are the most potent inducers of *nod* gene expression in *B. japonicum* (5, 19). In a recent study, these compounds as well as other isoflavones were shown not to be chemoattractants of *B. japonicum* (18). In contrast, *Rhizobium meliloti*, *Rhizobium leguminosarum* biovar viciae, and *R. leguminosarum* biovar phaseoli all showed chemotaxis to flavonoid inducers of their respective nodulation genes (2, 4, 10). Mutations in the *nodA*, *nodC*, and *nodD* genes of *R. meliloti* abolished chemotaxis to the flavonoid luteolin but did not affect general chemotaxis of the bacteria. Absence of the symbiotic megaplasmid in *R. leguminosarum* biovar viciae resulted in a reduced, but still positive, chemotactic response to the *nod* gene inducers naringenin and apigenin (4).

In the present study, soybean seed and root exudate and a range of pure compounds including isoflavones were tested for their ability to serve as chemoattractants for *B. japonicum*. *nod* gene mutants were also tested to determine the role of these genes in chemotaxis of *B. japonicum*. The concentrations of selected attractants in seed and root exudate were determined in an effort to identify possible natural chemoattractants.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *B. japonicum* strains were maintained on RDY agar as described previously (22). Strains used in chemotaxis assays were *B. japonicum* USDA 110 and transposon Tn*5* mutants of USDA 110 in the *nodY* (i.e., strain AN-277), the *nodC* (i.e., strain AN-308), or the *nodD* (i.e., strain AN-314) gene (22).

Chemotaxis assay. The capillary assay of Adler (1) was used to measure chemotaxis. Bacteria were cultured in minimal medium (MM) (6) with glycerol as a carbon source and glutamate as a nitrogen source. Mid-log-phase cultures were harvested by centrifugation at 4,000 × *g*, washed in MM without glutamate (MM−G), and then resuspended in the same medium to an optical density at 600 nm of 0.02 to 0.03. Attractants were prepared in MM−G, loaded into 1-μl capillaries, and placed in the bacterial suspensions. After 1 h, the capillaries were rinsed with distilled water and their contents were ejected into dilution tubes and plated at three dilutions. Three replications per treatment were used, and each treatment was repeated in at least two independent experiments. More variability was observed between experiments than between replicate assays within a single experiment. Relative responses were calculated as the number of CFU per capillary containing a test compound divided by the number of CFU in capillaries containing MM−G (controls). The average number of CFU for control capillaries was approximately 10^5. Controls in which attractants (succinate or daidzein) were added to both bacterial suspensions and capillaries showed no chemotaxis.

Preparation of exudates and exudate fractions. Soybean seed exudate was prepared by soaking *Glycine max* cv. Essex seeds in 1 ml of water or 50% ethanol per seed for 18 h and concentrating the aqueous portion 10-fold by rotary evaporation. In some cases, the seed exudate was used without concentration. Soybean root exudate was prepared by soaking 2-day-old seedlings in 4 ml of water per seedling for 4 days and concentrating the aqueous portion by lyophilization. Both exudates were sterilized by filtration. For the seed exudate fractionation experiment, 1 ml of the exudate was loaded onto a SepPak C_{18} column (Waters Associates, Inc., Milford, Mass.), the flowthrough fraction was col-

* Corresponding author.
† Present address: DuPont Haskell Laboratory, Newark, DE 19714.
‡ Present address: Landis International Inc., Valdosta, GA 31603-5126.

TABLE 1. Chemotactic response of *B. japonicum* USDA 110 to soybean exudates and selected flavonoids

Compound[a]	Relative response[b]
Seed exudate (0.1×)[c]	46.0 ± 4.4
Root exudate (10×)[d]	53.0 ± 8.1
Genistein	2.1 ± 0.5
Daidzein	2.2 ± 0.5
Daidzin[c]	3.2 ± 0.4
Luteolin	2.7 ± 0.5

[a] Pure compounds were tested at 10^{-6} M.
[b] Values are means ± standard error.
[c] Seed and root exudates at 1× are at the original strength (1 ml per seed or 4 ml per root) before concentration or dilution.
[d] Data were derived from a single experiment with three replications per treatment.

TABLE 3. Chemotactic response of *B. japonicum* USDA 110 *nod* gene mutants to soybean seed exudate and genistein

Strain	Relative response[a]	
	Seed exudate (0.1×)[b]	Genistein (10^{-6} M)
USDA 110	47.6 ± 9.1	1.2 ± 0.1
AN-277 (*nodY*-Tn*5*)	35.5 ± 0.6	1.6 ± 0.3
AN-308 (*nodC*-Tn*5*)	41.3 ± 5.9	1.0 ± 0.2
AN-314 (*nodD*-Tn*5*)	40.1 ± 3.9	1.4 ± 0.2

[a] Data were derived from a single experiment with three replications per treatment.
[b] See footnote *c* of Table 1.

lected, and then the column was washed with 4 ml of water and eluted with 100% ethanol. The flowthrough and 100% ethanol fractions were lyophilized and then resuspended in 1 ml of MM−G. Previous work has shown the 100% ethanol fraction to contain genistein and daidzein as well as glycosylated derivatives of these isoflavones (25).

Induction of *nod* genes. *B. japonicum* mutant strains were induced with soybean seed exudate as described previously (5). Strain ZB977 (USDA 110 carrying a *nodY-lacZ* fusion) (5) was used as a positive control for induction.

Biochemical analyses. Amino acid analyses of seed and root exudate were performed by using the Picotag high-pressure liquid chromatography system (Waters) and procedures described by the manufacturer. Succinate and malate concentrations in exudates were determined enzymatically by using specific biochemical analysis kits (Boehringer Mannheim, Inc., Indianapolis, Ind.).

RESULTS

Chemotaxis to exudates and flavonoids. *B. japonicum* USDA 110 was found to be strongly chemotactic toward soybean seed and root exudates (Table 1). The isoflavones genistein, daidzein, and daidzin, which induce *nod* gene transcription in USDA 110 (5), were found to be very weak attractants. These three compounds are the predominant *nod* gene inducers found in soybean seed extract and root exudates (25). Relative responses for genistein were determined over a range of concentrations and were found to be the highest (approximately 2 to 3) at concentrations between 10^{-5} and 10^{-7} M (Table 1 and data not shown). The flavone luteolin which is not a *nod* gene inducer of USDA 110 (5) was also a weak attractant.

Table 2 shows the results of an experiment with seed exudate which was separated into crude fractions based on polarity. The flowthrough fraction (more polar) was found to contain approximately 87% of the chemotactic activity (relative response of 32.0) but only 33% of the *nodY*-inducing activity (349 U). Conversely, the 100% ethanol fraction (more nonpolar) contained approximately 77% of the *nodY*-inducing activity recovered (1,192 U) but only 13% of the chemotactic activity (relative response of 4.9). As expected, the relatively nonpolar flavonoid compounds were mostly confined to the 100% ethanol fraction. On the other hand, most of the chemotactic activity was represented by the more-polar flowthrough fraction.

TABLE 2. Chemotactic response and *nod* gene induction activity of fractionated soybean seed exudate

Fraction[a]	Relative response[b]	β-Galactosidase activity (U)
Total exudate	46.0 ± 3.9	1870 ± 147
Flowthrough	32.0 ± 2.1	349 ± 59
100% Ethanol	4.8 ± 0.6	1192 ± 2
Flowthrough + 100% ethanol	31.3 ± 2.4	1631 ± 36

[a] Exudates were fractionated by using a SepPak C18 cartridge (Waters).
[b] Values are means ± standard error.

B. japonicum USDA 110 strains with mutations in either *nodY*, *nodC*, or *nodD* all showed wild-type chemotaxis to seed exudate (Table 3). In another experiment (data not shown), chemotaxis of cells induced with either seed exudate or genistein was found to be the same as chemotaxis of uninduced cells when seed exudate, genistein, or succinate was used as an attractant.

Chemotaxis to amino acids, organic acids, and sugars. Since the flavonoid components did not account for the strong chemoattraction of *B. japonicum* to soybean seed and root exudate, other components were tested. Of 14 amino acids, only glutamate and aspartate were strong attractants, showing relative responses of 23.6 and 15.0, respectively (Table 4). All other amino acids showed low, but still positive, relative responses (1.7 to 4.4). Table 5 shows chemotactic responses to organic acids and sugars. Seven of eight organic acids were found to be attractants, while none of the six sugars tested was a significant attractant.

Amino acid and organic acid concentrations in exudates. The concentrations of various amino acids in seed and root exudate are shown in Table 4. The same four amino acids, glutamate, aspartate, alanine, and arginine, were found in higher concentrations than the other amino acids in both exudates. The amino acid concentrations were more than 10-fold less in the 1× root exudate than in the 1× seed exudate. Of course these numbers are not intended to reflect any natural situation, since the exudates were prepared in arbitrary volumes (1 ml per seed [1× root exudate] and 4 ml per seedling [1× seed exudate]). To determine whether the chemotaxis shown to organic acids was relevant, the concentrations of succinate and malate in seed and root exudates were determined and the concentration response for chemotaxis to these compounds was tested. Malate was found to be present at 0.24 and 0.14 mM in seed and root exudates, respectively, while less than 0.1 mM succinate (below the limit of accurate quantification) was present in both exudates. Figure 1A shows concentration response curves for glutamate and succinate. Chemotaxis to glutamate increased sharply between 10^{-3} and 10^{-1} M (the highest concentration tested), while glutamate showed weak

TABLE 4. Chemotactic response of *B. japonicum* USDA 110 to amino acids present in soybean seed and root exudate

Amino acid	Relative response[a]	Concn (mM)[b]	
		Seed exudate	Root exudate
Glutamate	23.6 ± 4.6	4.45	0.149
Aspartate	15.0 ± 3.2	4.38	0.252
Alanine	4.4 ± 0.5	2.41	0.153
Cysteine	3.9 ± 0.8		
Proline	3.8 ± 0.8	1.52	0.030
Serine	3.3 ± 0.5	2.21	0.093
Histidine	3.1 ± 0.4	0.76	0.066
Methionine	2.5 ± 0.5	1.34	0.009
Arginine	2.3 ± 0.5	4.26	0.159
Glycine	2.2 ± 0.5	2.73	0.035
Tyrosine	2.1 ± 0.0	1.22	0.006
Leucine	1.9 ± 0.3	1.36	0.041
Valine	1.7 ± 0.4	1.31	0.070
Lysine	ND[c]	1.24	0.010
Threonine	ND	1.17	0.077
Phenylalanine	ND	0.92	0.031
Isoleucine	ND	0.76	0.041

[a] Values were obtained at 10 mM each amino acid tested. Each value is a mean ± standard error.
[b] Concentrations are representative of 1× seed and root exudates (see Table 1, footnote *c*).
[c] ND, not determined.

attraction at concentrations of 10^{-3} M or lower. On the other hand, succinate showed a maximum response at a concentration of 10^{-3} M and a weak response at a concentration as low as 10^{-5} M. Likewise, malate was found to be an attractant at a concentration as low as 10^{-4} M (relative response of 7.8). Figure 1B shows response curves for dilutions of soybean seed and root exudates. A 10-fold dilution of seed exudate gave a maximum response, while the chemotactic response to root exudate increased up to the highest concentration tested.

DISCUSSION

Chemotaxis in *Rhizobium* spp. has been the subject of a number of studies, some of which have described the attraction of the bacteria to root surfaces or host plant exudates (12, 13, 14). In this study, soybean seed and root exudates were shown to be potent chemoattractants for *B. japonicum*.

TABLE 5. Chemotactic response of *B. japonicum* USDA 110 to selected organic acids and sugars

Compound	Relative response[a]
Organic acids	
Succinate	25.4 ± 3.0
Malonate	24.0 ± 1.2
Malate	17.0 ± 1.5
α-Ketoglutarate	15.5 ± 3.1
Fumarate	14.4 ± 1.1
Pyruvate	12.5 ± 2.2
Citrate	3.1 ± 0.4
Sugars	
Mannitol	3.0 ± 0.6
Arabinose	1.9 ± 0.4
Galactose	1.7 ± 0.3
Sucrose	1.4 ± 0.2
Glucose	1.3 ± 0.2

[a] Values are means ± standard error.

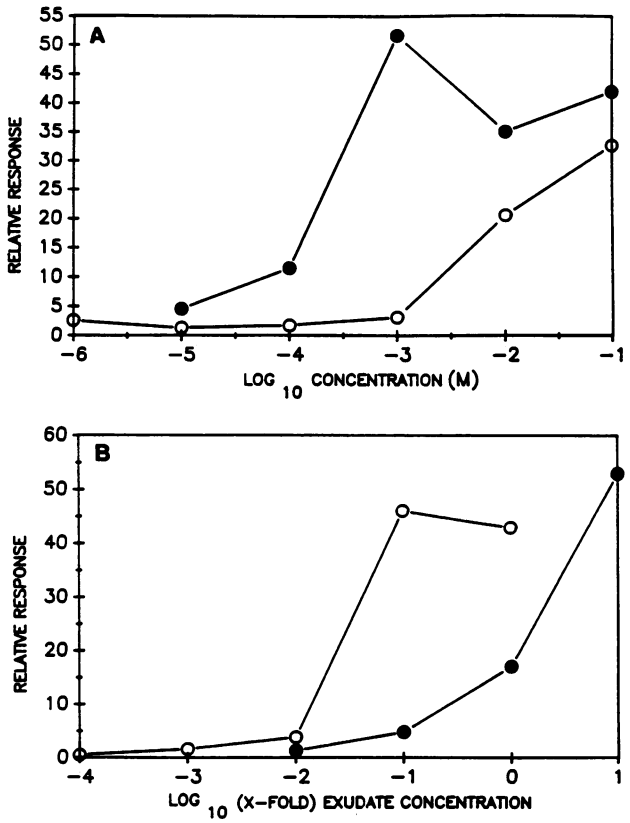

FIG. 1. Relative response curves of *B. japonicum* to various concentrations of glutamate (○) and succinate (●) (A) or soybean seed exudate (○) and soybean root exudate (●) (B). Each value is the mean of three replicates from a single experiment. Standard errors were, on average, 15% of their respective means.

Since exudates are known to contain a wide range of organic compounds (11, 13), it was not surprising that attractants were present.

In an effort to determine what chemicals were attractants of *B. japonicum*, a range of compounds were screened by capillary assay, including amino acids, sugars, and organic acids. Of these compounds, only the amino acids glutamate and aspartate and seven of the eight organic acids tested showed relative responses of greater than 5. All of the strong attractants identified, with the exception of pyruvate, are dicarboxylic acids, indicating that this class of compound may represent important attractants for *B. japonicum*. This is supported by the fact that some of these compounds were found to be present in soybean exudates in concentrations sufficient to elicit a strong chemotactic response (e.g., glutamate and malate). The concentrations determined in this study were obtained from seeds or roots placed in a relatively large amount of liquid, and, therefore, the natural concentrations of these chemicals at the root surface could be significantly higher. Several compounds including amino acids, organic acids, and sugars have been shown to be attractants of *Rhizobium* spp. (2, 7, 13, 15). The apparent broad specificity for chemoattractants of *Rhizobium* spp. is in contrast with the more narrow class of *B. japonicum* attractants identified in the present study. It should be noted, however, that aromatic and hydroaromatic acids (23) and hydroxycinnamic acids (18) have also been shown to be

attractants. In the study of aromatic and hydroaromatic acids, no relative response values were reported, while the relative responses for hydroxycinnamic acids were less than 10. Neither study correlated chemotaxis to concentration of the attractants in soybean exudates.

Recent reports have described the attraction of *Rhizobium* spp. to the flavonoids which specifically induce transcription of the *nod* genes (2, 4, 10). In light of these reports and the fact that flavonoids are also determinants in *nod* gene expression in *Bradyrhizobium* spp., it was important to assess to what extent the attraction to the seed and root exudates could be attributed to flavonoids contained in the exudates. Recently, Kape et al. (18) reported that *B. japonicum* showed no chemotaxis to the isoflavones genistein and daidzein. In the present study, these compounds were found to be weak attractants. Two lines of evidence from this study suggest that, although these compounds can serve as chemoattractants, they are not major attractants in the exudates. First, capillary assays revealed that the isoflavones were relatively weak attractants when compared with the exudates. Second, separation of a flavonoid fraction from the total seed exudate resulted in a loss of only 13% of the exudate chemotactic activity, but a loss of nearly 80% of the *nod* gene inducing activity, indicating that the primary chemotactic components and the primary inducing components are chemically separate. The relevance of the weak chemotactic responses found for isoflavones (relative responses of less than 3.0) is not known. Caetano-Anolles et al. (10) showed maximum relative responses of approximately 2 for attraction to luteolin and concluded that such responses were potentially important. On the other hand, relative responses of more than 10 for attraction of *R. leguminosarum* biovar viciae and *R. leguminosarum* biovar phaseoli to their respective *nod* gene inducers have been demonstrated (2, 4).

Mutations in *nodY*, *nodC*, or *nodD* did not substantially affect chemotaxis to seed exudate or the isoflavone genistein. In contrast, earlier reports showed that chemotaxis to luteolin by *R. meliloti* was eliminated by mutation of the *nodD*, *nodA*, or *nodC* genes (10), while chemotaxis to apigenin and naringenin by *R. leguminosarum* biovar viciae was significantly reduced in a Sym plasmid-cured mutant (4). Thus, these differences may reflect a fundamental difference between *Rhizobium* and *Bradyrhizobium* species.

Since the common *nod* genes are expressed at a low level (*nodD*) or are not expressed (e.g., *nodYABC*) in the absence of isoflavones (5), it seemed necessary to determine whether a chemotactic response to isoflavones was affected by *nod* gene induction. Pretreatment of cells with seed exudate or genistein to induce *nod* gene expression did not affect the chemotactic response of *B. japonicum*.

Given the weak chemotactic response of *B. japonicum* to isoflavones, it would appear unlikely that these chemicals are important natural attractants. Instead, dicarboxylic acids (including amino acids glutamate and aspartate) appear to be potential candidates for natural attractants. However, it is quite possible that a variety of compounds in soybean exudates serve as natural attractants.

ACKNOWLEDGMENTS

This work was supported by Public Health Service grants GM 33494 and GM 40183 from National Institutes of Health and grant 88-37262-3894 from the U.S. Department of Agriculture.

REFERENCES

1. **Adler, J.** 1973. A method for measuring chemotaxis and use of the method to determine optimum conditions for chemotaxis by *Escherichia coli*. J. Gen. Microbiol. **74**:77–91.
2. **Aguilar, M. M., A. M. Ashby, A. J. M. Richards, G. J. Loake, M. D. Watson, and C. H. Shaw.** 1988. Chemotaxis of *Rhizobium leguminosarum* biovar phaseoli towards flavonoid inducers of the symbiotic nodulation genes. J. Gen. Microbiol. **134**:2741–2746.
3. **Ames, P., and K. Bergman.** 1981. Competitive advantage provided by bacterial mobility in the formation of nodules by *Rhizobium meliloti*. J. Bacteriol. **148**:728–729.
4. **Armitage, J. P., A. Gallagher, and A. W. B. Johnston.** 1988. Comparison of the chemotactic behavior of *Rhizobium leguminosarum* with and without the nodulation plasmid. Mol. Microbiol. **2**:743–748.
5. **Banfalvi, Z., A. Nieuwkoop, M. Schell, L. Besl, and G. Stacey.** 1988. Regulation of *nod* gene expression in *Bradyrhizobium japonicum*. Mol. Gen. Genet. **214**:420–424.
6. **Bergersen, F. J.** 1961. The growth of rhizobia in synthetic media. Aust. J. Biol. Sci. **14**:349–360.
7. **Bergman, K., M. Gulash-Hoffee, R. E. Hovestadt, R. C. Larosiliere, R. G. Ronco II, and L. Su.** 1988. Physiology of behavioral mutants of *Rhizobium meliloti*: evidence for a dual chemotaxis pathway. J. Bacteriol. **170**:3249–3254.
8. **Bhuvaneswari, T. V., A. A. Bhagwat, and W. D. Bauer.** 1981. Transient susceptibility of root cells in four common legumes to nodulation by rhizobia. Plant Physiol. **68**:1144–1149.
9. **Bhuvaneswari, T. V., B. G. Turgeon, and W. D. Bauer.** 1980. Early events in the infection of soybean (*Glycine max* L. Merr) by *Rhizobium japonicum*. I. Location of infectible root cells. Plant Physiol. **66**:1027–1031.
10. **Caetano-Anolles, G., D. K. Crist-Estes, and W. D. Bauer.** 1988. Chemotaxis of *Rhizobium meliloti* to the plant flavone luteolin requires functional nodulation genes. J. Bacteriol. **170**:3164–3169.
11. **Curl, E. A., and B. Truelove (ed.).** 1986. Root exudates, p. 55–92. *In* The rhizosphere. Springer-Verlag, Berlin.
12. **Currier, W. W., and G. A. Strobel.** 1977. Chemotaxis of *Rhizobium* spp. to a glycoprotein produced by Birdsfoot trefoil roots. Science **196**:434–436.
13. **Gaworzewska, E. T., and M. J. Carlile.** 1982. Positive chemotaxis of *Rhizobium leguminosarum* and other bacteria towards root exudates from legumes and other plants. J. Gen. Microbiol. **128**:1179–1188.
14. **Gitte, R. R., P. Vittal Rai, and R. B. Patil.** 1978. Chemotaxis of *Rhizobium* sp. towards root exudate of *Cicer arietinum* L. Plant Soil **50**:553–566.
15. **Gotz, R., N. Limmer, K. Ober, and R. Schmitt.** 1982. Motility and chemotaxis in two strains of *Rhizobium* with complex flagella. J. Gen. Microbiol. **128**:789–798.
16. **Gulash, M., P. Ames, R. C. Larosiliere, and K. Bergman.** 1984. Rhizobia are attracted to localized sites on legume roots. Appl. Environ. Microbiol. **48**:149–152.
17. **Hunter, W. J., and C. J. Fahring.** 1980. Movement by *Rhizobium* and nodulation of legumes. Soil Biol. Biochem. **12**:537–542.
18. **Kape, R., M. Parniske, and D. Werner.** 1991. Chemotaxis and *nod* gene activity of *Bradyrhizobium japonicum* in response to hydroxycinnamic acids and isoflavonoids. Appl. Environ. Microbiol. **57**:316–319.
19. **Kosslak, R. M., R. Bookland, J. Barkei, H. Paaren, and E. R. Appelbaum.** 1987. Induction of *Bradyrhizobium japonicum* common *nod* genes by isoflavones isolated from *Glycine max*. Proc. Natl. Acad. Sci. USA **84**:7428–7432.
20. **Long, S. R.** 1989. *Rhizobium*-legume nodulation: life together in the underground. Cell **56**:203–214.
21. **Mellor, H. Y., A. R. Glenn, R. Arwas, and M. J. Dilworth.** 1987. Symbiotic and competitive properties of motility mutants of *Rhizobium trifolii* TA1. Arch. Microbiol. **148**:34–39.
22. **Nieuwkoop, A. J., Z. Banfalvi, N. Deshmane, D. Gerhold, M. G. Schell, K. M. Sirotkin, and G. Stacey.** 1987. A locus encoding host range is linked to the common nodulation genes of

Bradyrhizobium japonicum. J. Bacteriol. **169**:2631–2638.

23. **Parke, D., M. Rivelli, and L. N. Ornston.** 1985. Chemotaxis to aromatic and hydroaromatic acids: comparison of *Bradyrhizobium japonicum* and *Rhizobium trifolii*. J. Bacteriol. **163**:417–422.
24. **Porter, P. M., W. L. Banwart, and J. J. Hassett.** 1985. HPLC isolation and GC-MS identification of genistein, daidzein, and coumestrol from unhydrolysed soybean root extracts. Environ. Exp. Bot. **25**:229–232.
25. **Smit, G., V. Puvanesarajah, R. Carlson, and G. Stacey.** 1990. *Bradyrhizobium japonicum nodD* can specifically be induced by soybean seed extract compounds which do not induce the *nodYABC* genes, p. 274. *In* P. M. Gresshoff, L. E. Roth, G. Stacey, and W. E. Newton (ed.), Nitrogen fixation: achievements and objectives. Chapman & Hall, Ltd., London.
26. **Soby, S., and K. Bergman.** 1983. Motility and chemotaxis of *Rhizobium meliloti* in soil. Appl. Environ. Microbiol. **46**:995–998.