Chemotaxis of *Rhizobium leguminosarum* biovar *phaseoli* towards Flavonoid Inducers of the Symbiotic Nodulation Genes

By J. MANUEL MUNOZ AGUILAR,¹ ALISON M. ASHBY,¹
ANDREW J. M. RICHARDS,² GARY J. LOAKE,¹
MARTIN D. WATSON¹ AND CHARLES H. SHAW¹*

¹ Department of Botany, University of Durham, Science Laboratories, South Road,
Durham DH1 3LE, UK

² ICI Biological Products Business, PO Box 1, Billingham, Cleveland, TS23 1LB, UK

(Received 28 March 1988; revised 2 June 1988)

Chemotaxis of *Rhizobium leguminosarum* biovar *phaseoli* RP8002 towards a range of carbohydrates, phenolic compounds and flavonoids was assayed. Xylose (peak response 10^{-4} M), sucrose (peak response 10^{-6} M) and raffinose (peak response 10^{-5} M) were strong chemoattractants amongst the carbohydrates, whilst glucose, fructose, galactose and maltose produced little or no detectable response. Of the monocyclic phenolic compounds, vanillyl alcohol, *p*-hydroxybenzoic acid (both peak responses 10^{-6} M) and 3,4-dihydroxybenzoic acid (peak response 10^{-4} M) all evoked strong chemotactic responses. Amongst the *nod*-inducing flavonoids, apigenin and luteolin were both strong chemoattractants (peaks at 10^{-5} M) while naringenin produced a very low response. Competition experiments suggest that apigenin and luteolin are recognized by a common receptor, but that there exists a separate receptor for luteolin alone. The inhibitors of *nod*-induction, umbelliferone and acetosyringone, both produced strong chemotactic responses, with peaks at 10^{-3} M and 10^{-2} M respectively. This evidence is indicative of a role for chemotaxis towards *nod*-inducing flavonoids in the initiation of root nodule formation by rhizobia, and also suggests that chemotaxis may influence the host range of the interaction.

INTRODUCTION

The formation of nitrogen-fixing nodules by *Rhizobium* upon legume roots is a complex and highly organized process. The nodulation (*nod*) functions are usually clustered on large (sym) plasmids in the microbial symbiont (Rossen *et al.*, 1987). Flavonoids in legume root exudates interact with the *nodD* product to effect induction of the other *nod* genes (Peters *et al.*, 1986; Redmond *et al.*, 1986; Firmin *et al.*, 1986; Zaat *et al.*, 1987; Horvath *et al.*, 1987; Djordjevic *et al.*, 1987; Kosslak *et al.*, 1987). The exact functions of the induced *nod* genes are unclear, although *nodA*, *B* and *C* are required for root hair curling and mutations in the other *nod* loci affect subsequent nodule formation (Rossen *et al.*, 1987).

A similar relationship exists between *Agrobacterium tumefaciens* and susceptible plants. Wound exuded phenolic compounds, e.g. acetosyringone, induce the virulence (*vir*) operons on the Ti-plasmid, via an interaction with the products of *virA* and *G* (Okker *et al.*, 1984; Stachel *et al.*, 1985, 1986; Stachel & Zambryski, 1986; Winans *et al.*, 1986; Rogowsky *et al.*, 1987). The induced *vir* functions mediate T-DNA transfer to the plant cell; expression of the integrated T-DNA leads to proliferation of cells to form a crown gall tumour (Nester *et al.*, 1984; Lichtenstein, 1986). Ti-plasmid harbouring strains of *A. tumefaciens* are chemotactic towards *vir*-inducing phenolic compounds, such as acetosyringone (Ashby *et al.*, 1987, 1988). This

Abbreviations: PHBA, *p*-hydroxybenzoic acid; DHBA, 3,4-dihydroxybenzoic acid.

response requires *virA* and *G* (Shaw *et al.*, 1988) and has a threshold of $<10^{-8}$ M (Ashby *et al.*, 1987). This suggests that chemotaxis to *vir*-inducing wound exudates is responsible for attracting and guiding virulent *A. tumefaciens* towards susceptible cells (Shaw *et al.*, 1986).

Agrobacterium and *Rhizobium* exhibit similar motility patterns (Götz & Schmitt, 1987; Loake *et al.*, 1988). *Rhizobia* are chemotactic to a variety of amino acids and sugars (Götz *et al.*, 1982; Gaworzewska & Carlile, 1982). Moreover, attraction to a legume glycoprotein (Currier & Strobel, 1976, 1977), root exudates (Currier & Strobel, 1976; Gitte *et al.*, 1978; Gaworzewska & Carlile, 1982) and localized sites upon legume roots (Gulash *et al.*, 1984) has been reported for *Rhizobium*. Furthermore, motile strains of *R. meliloti* appear to have an advantage in nodule formation over non-motile mutants (Ames & Bergman, 1981).

In view of the similarities between *Agrobacterium* and *Rhizobium*, we undertook an investigation of chemotaxis in *Rhizobium leguminosarum* biovar *phaseoli*.

METHODS

Organism and growth. All experiments were done with *R. leguminosarum* biovar *phaseoli* RP8002, because in comparative experiments it was significantly more motile than three other biovars of *R. leguminosarum* (JIINT83K3, RL1040 and RL16015). Bacteria were grown either in *Rhizobium* Initiation Medium (RIM; 0.5% tryptone, 0.3% yeast extract, 0.13% CaCl_2) or Minimal A supplemented with glucose (MinA + G; Miller, 1972). Periodically, a fresh isolate of motile bacteria was prepared from the stock culture by three passages through swarm plates (RIM solidified with 0.25% agar), selecting organisms from the perimeter of the swarm at each passage. Chemotactic responses of successive isolates were reproducible. Chemotaxis medium (CM; Adler, 1973) was autoclaved and attractants were filter sterilized prior to use.

Chemotaxis assay. Capillary assays (Adler, 1973) were done in duplicate, using washed stationary phase cells at a culture density of approximately 10^9 cells ml^{-1} , as previously described (Ashby *et al.*, 1987). Bacteria from the exponential phase gave qualitatively similar, but quantitatively lower responses. Values presented represent the mean of duplicate assays done on at least two separate occasions. All values recorded fell within 20% of the mean.

Microscopy. Motility was observed at room temperature, using a Nikon Optiphot microscope with phase-contrast optics, and a Hitachi HV-720K CCTV camera. Behaviour and speed measurements were made during slow motion playback of recorded motility, using a Ferguson 3V23 video recorder and a Hitachi VM-920K video monitor.

Attractants. All sugars were obtained from Sigma, flavonoids from Apin Chemicals (Oxford, UK) and phenolic compounds from Aldrich. Phenolic compounds were solubilized by neutralization with KOH, and flavonoids in a 1:1 (v/v) mixture of acetonitrile and dimethylsulphoxide, neither of which are chemoattractants for *R. leguminosarum* biovar *phaseoli*.

RESULTS

Chemotaxis towards sugars

A variety of mono-, di- and trisaccharides were tested as chemoattractants for *R. leguminosarum* biovar *phaseoli* using the capillary assay (Adler, 1973; Ashby *et al.*, 1987). Of the monosaccharides tested (Fig. 1) only xylose evoked a chemotactic response, yielding a relatively broad peak with an apparent optimum of 10^{-4} M. No significant chemotaxis was recorded towards fructose, galactose or glucose. Sucrose (peak chemotaxis at 10^{-6} M) and the trisaccharide raffinose (peak at 10^{-5} M), evoked a significant response (Fig. 2) while a barely detectable response was produced by maltose.

Chemotaxis towards phenolic compounds

In view of our recent demonstration of the significance of chemotaxis of *A. tumefaciens* towards phenolic plant exudates (Ashby *et al.*, 1987, 1988; Shaw *et al.*, 1988) it was important to ascertain whether *R. leguminosarum* biovar *phaseoli* exhibited similar responses. All four compounds tested evoked some form of chemotactic response (Fig. 3). Vanillyl alcohol and *p*-hydroxybenzoic acid (PHBA) both gave sharp peaks at 10^{-6} M, 3,4-dihydroxybenzoic acid (DHBA) resulted in a broad peak with an apparent maximum at 10^{-4} M, and acetosyringone evoked a response which increased towards 10^{-2} M. Solubility problems above that concentration prevented a proper peak from being observed. These results are at considerable variance to those recorded for *A. tumefaciens* (see Discussion). It is of interest to note that acetosyringone is an inhibitor of *nod*-induction in *R. leguminosarum* (Firmin *et al.*, 1986).

Fig. 1

Fig. 2

Fig. 1. Dose response curves of motility of *R. leguminosarum* biovar *phaseoli* towards the monosaccharides xylose (○), fructose (●), galactose (□) and glucose (■). ---, Number of bacteria attracted into a capillary containing chemotaxis medium alone.

Fig. 2. Dose response curves of motility of *R. leguminosarum* biovar *phaseoli* towards the oligosaccharides sucrose (□), maltose (●) and raffinose (■). ---, As Fig. 1.

Fig. 3

Fig. 4

Fig. 3. Dose response curves of motility of *R. leguminosarum* biovar *phaseoli* towards the phenolic compounds (a) acetosyringone (○) and vanillyl alcohol (●), and (b) PHBA (○) and DHBA (●). ---, As Fig. 1.

Fig. 4. Dose response curves of motility of *R. leguminosarum* biovar *phaseoli* towards the flavonoids apigenin (○), luteolin (●), naringenin (□) and umbelliferone (■). Control values of bacteria attracted into a capillary containing chemotaxis medium alone were $4.5 \times 10^6 \text{ ml}^{-1}$.

Table 1. *Competitive chemotaxis assays for R. leguminosarum, biovar phaseoli*

Attractant in capillary (10^{-5} M)	Attractant in pool (10^{-4} M)	$10^{-6} \times$ No. of cells ml^{-1} in capillary
Chemotaxis medium	—	8.7
Apigenin	—	28.3
Luteolin	—	29.4
Apigenin + luteolin	—	38.6
Apigenin + luteolin	Apigenin	22.1
Apigenin + luteolin	Luteolin	6.9

Chemotaxis towards flavonoids

Due to their significance as *nod*-inducers, it was important to test chemotaxis towards flavonoids. Both apigenin and luteolin, which are *nod*-inducers for *R. meliloti*, *R. leguminosarum* and *R. trifolii* (Peters *et al.*, 1986; Firmin *et al.*, 1986; Zaat *et al.*, 1987; Djordjevic *et al.*, 1987) are potent chemoattractants, producing sharp peaks at 10^{-5} M (Fig. 4). In contrast, naringenin, a *nod*-inducer for *R. leguminosarum* and *R. trifolii* (Zaat *et al.*, 1987; Djordjevic *et al.*, 1987) evoked a very weak response. Intriguingly, umbelliferone, which has been reported to be an inhibitor of *nod*-induction in *R. trifolii* (Djordjevic *et al.*, 1987), acted as a strong chemoattractant for *R. leguminosarum* biovar *phaseoli*, exhibiting a peak at 10^{-3} M.

As apigenin and luteolin are structurally similar, competition experiments were done to ascertain whether they share a receptor (Table 1). At 10^{-5} M both flavonoids evoked a strong chemotactic response, which was marginally improved when the two compounds were used together as attractant. This response was slightly attenuated by the inclusion, in the bacterial pool, of 10^{-4} M-apigenin. However, when 10^{-4} M-luteolin was included in the bacterial pool, chemotaxis towards the apigenin/luteolin mixture dropped below the level of detection. These results indicate that saturation with luteolin blocks chemotaxis towards apigenin, but apigenin at saturation only partially impedes chemotaxis towards luteolin. This suggests that there exist in *R. leguminosarum* biovar *phaseoli* two separate receptor/binding sites for luteolin, one of which also recognizes apigenin.

DISCUSSION

This paper is the first survey of chemotaxis in *Rhizobium leguminosarum* biovar *phaseoli*, and illustrates the significant differences between Rhizobia. The response of *R. leguminosarum* biovar *phaseoli* towards carbohydrates is almost completely different to the recorded behaviour of other biovars of *R. leguminosarum*, *meliloti* and *lupini* (Götz *et al.*, 1982; Gaworzewska & Carlile, 1982). Xylose is also an attractant for *R. leguminosarum*, and glucose fails to attract *R. meliloti*, but all other responses of *R. leguminosarum* biovar *phaseoli* are distinct. What is also distinct about the *R. leguminosarum* biovar *phaseoli* responses is their greater sensitivity than in other Rhizobia (Götz *et al.*, 1982; Gaworzewska & Carlile, 1982). Sucrose, for example, produces a peak response at 10^{-6} M, reminiscent of the behaviour of *A. tumefaciens* (Loake *et al.*, 1988). It is quite intriguing that *R. leguminosarum* biovar *phaseoli* is not chemotactic towards some sugars (glucose, fructose and maltose) which are major constituents of legume root exudates (Gaworzewska & Carlile, 1982) but is attracted towards other important components of these exudates (xylose, sucrose and raffinose).

Chemotaxis towards flavonoids and phenolic compounds is particularly interesting. The responses towards phenolic compounds are significantly different to those of *A. tumefaciens* (Ashby *et al.*, 1987, 1988). PHBA and DHBA are both chemoattractants for *A. tumefaciens* (Ashby *et al.*, 1988), non-inducers of the *nod* genes, and degradation products of flavones (Djordjevic *et al.*, 1987). *A. tumefaciens* is also chemotactic to non-*vir*-inducing phenolic compounds such as vanillyl alcohol (Ashby *et al.*, 1987). Non-inducing attractants, such as sugars and some phenolic compounds, may thus serve to guide *Agrobacterium* and *Rhizobium* to the vicinity of plant roots.

Both apigenin and luteolin are strong chemoattractants for *R. leguminosarum* biovar *phaseoli*, and *nod*-inducers for *R. meliloti*, *R. leguminosarum* biovar *viciae* and *R. trifolii* (Peters *et al.*, 1986; Firmin *et al.*, 1986; Zaat *et al.*, 1987; Djordjevic *et al.*, 1987). The chemotactic response is highly sensitive, and within the same concentration range as that evoking maximal *nod*-induction in *R. trifolii* (Djordjevic *et al.*, 1987). Intriguingly, naringenin, closely related and also a *nod*-inducer for *R. leguminosarum* biovar *viciae* and *R. trifolii* (Zaat *et al.*, 1987; Djordjevic *et al.*, 1987) failed to elicit a significant chemotactic response. These responses may play a role in the determination of rhizobial host range. It has recently been demonstrated that apigenin and naringenin, but not luteolin, are good *nod*-inducers in *R. leguminosarum* biovar *phaseoli* (Carmen Quinto, personal communication). Thus, our results indicate that chemotaxis and *nod*-induction do not necessarily correlate. A similar conundrum is observed in *R. meliloti*, where some *nod*-inducers, such as luteolin, do act as chemoattractants, but others, such as apigenin and naringenin, do not, and indeed the latter suppresses chemotaxis towards luteolin (Caetano-Anolles *et al.*, 1988). As legume root exudates are complex in composition (Gitte *et al.*, 1978; Gaworzewska & Carlile, 1982; Redmond *et al.*, 1986; Firmin *et al.*, 1986; Djordjevic *et al.*, 1987) the different spectra of compounds triggering chemotaxis and *nod*-induction may be part of an interaction which is significant in host range determination in *Rhizobium*.

The exaggerated response to high concentrations of acetosyringone and umbelliferone, both of which have been reported to be inhibitors of *nod*-induction (Firmin *et al.*, 1986; Djordjevic *et al.*, 1987) is intriguing. Djordjevic *et al.* (1987) proposed that the secretion of umbelliferone at or near the root tip in clover would prevent nodule initiation in this vicinity. However, it seems unlikely that the concentrations of these inhibitors required to evoke peak chemotactic responses in *R. leguminosarum* biovar *phaseoli* (10^{-2} to 10^{-3} M) would ever be encountered in the rhizosphere. Secretion of different attractants from distinct root zones could explain the attraction of *R. meliloti* to localized sites on legume roots (Gulash *et al.*, 1984).

This paper presents evidence suggestive of an involvement of chemotaxis towards flavonoids in the initiation of legume root nodules by rhizobia. Moreover, as different flavonoids evoke widely differing responses, there is a possibility that the specificity of chemotaxis is involved in the determination of rhizobial host range. In *A. tumefaciens* a similar relationship exists, and the same genetic loci are involved in acetosyringone mediated chemotaxis and *vir*-induction. It will therefore be of interest to elucidate the genetic basis of chemotaxis in *R. leguminosarum* biovar *phaseoli*.

The authors would like to express their gratitude to the members of the Durham *Agrobacterium* Group for continual advice and stimulating discussions. A.M.A. and G.J.L. were supported by SERC studentships.

REFERENCES

- ADLER, J. (1973). A method for measuring chemotaxis and use of the method to determine optimum conditions for chemotaxis by *Escherichia coli*. *Journal of General Microbiology* **74**, 77–91.
- AMES, P. & BERGMAN, K. (1980). Competitive advantage provided by bacterial motility in the formation of nodules by *Rhizobium meliloti*. *Journal of Bacteriology* **148**, 728–729.
- ASHBY, A. M., WATSON, M. D. & SHAW, C. H. (1987). A Ti-plasmid determined function is responsible for chemotaxis towards the plant wound product acetosyringone. *FEMS Microbiology Letters* **41**, 189–192.
- ASHBY, A. M., WATSON, M. D., LOAKE G. J. & SHAW, C. H. (1988). Ti-plasmid specified chemotaxis of *Agrobacterium tumefaciens* C58C¹ towards *vir*-inducing phenolics and soluble factors from monocotyledonous and dicotyledonous plants. *Journal of Bacteriology* (in the Press).
- CAETANO-ANOLLES, G., CRIST-ESTES, D. K. & BAUER, W. D. (1988). Chemotaxis of *Rhizobium meliloti* to the plant flavone luteolin requires functional nodulation genes. *Journal of Bacteriology* (in the Press).
- CURRIER, W. W. & STROBEL, G. A. (1976). Chemotaxis of *Rhizobium* spp. to plant root exudates. *Plant Physiology* **57**, 820–823.
- CURRIER, W. W. & STROBEL, G. A. (1977). Chemotaxis of *Rhizobium* spp. to a glycoprotein produced by birdsfoot trefoil roots. *Science* **196**, 434–436.
- DJORDJEVIC, M. A., REDMOND, J. A., BATLEY, M. & ROLFE, B. G. (1987). Clovers secrete specific phenolic compounds which either stimulate or repress *nod* gene expression in *Rhizobium trifolii*. *EMBO Journal* **6**, 1173–1179.
- FIRMIN, J. L., WILSON, K. E., ROSSEN, L. & JOHNSTON, A. W. B. (1986). Flavonoid activation of nodulation genes in *Rhizobium* reversed by other compounds present in plants. *Nature, London* **324**, 90–92.

- GAWORZEWSKA, E. T. & CARLILE, M. J. (1982). Positive chemotaxis of *Rhizobium leguminosarum* and other bacteria towards root exudates from legumes and other plants. *Journal of General Microbiology* **128**, 1179–1188.
- GITTE, R. R., RAI, P. V. & PATIL, R. B. (1978). Chemotaxis of *Rhizobium* spp. towards root exudate of *Cicer arietinum* L. *Plant and Soil* **50**, 553–566.
- GULASH, M., AMES, P., LAROSILIERE, R. C. & BERGMAN, K. (1984). Rhizobia are attracted to localised sites on legume roots. *Applied and Environmental Microbiology* **48**, 149–152.
- GÖTZ, R. & SCHMITT, R. (1977). *Rhizobium meliloti* swims by unidirectional, intermittent rotation of right-handed flagellar helices. *Journal of Bacteriology* **169**, 3146–3150.
- GÖTZ, R., LIMMER, N., OBER, K. & SCHMITT, R. (1982). Motility and chemotaxis in two strains of *Rhizobium* with complex flagella. *Journal of General Microbiology* **128**, 789–798.
- HORVATH, B., BACHEM, C. W. B., SCHELL, J. & KONDOROSI, A. (1987). Host-specific regulation of nodulating genes in *Rhizobium* is mediated by a plant signal, interacting with the *nodD* gene product. *EMBO Journal* **6**, 841–848.
- KOSSLAK, R. M., BOOKLAND, R., BARKEI, J., PAAREN, H. E. & APPELBAUM, E. R. (1987). Induction of *Bradyrhizobium japonicum* common *nod* genes by isoflavones isolated from *Glycine max*. *Proceedings of the National Academy of Sciences of the United States of America* **84**, 7428–7432.
- LICHTENSTEIN, C. (1986). A bizarre vegetal bestiality. *Nature, London* **322**, 682–683.
- LOAKE, G. J., ASHBY, A. M. & SHAW, C. H. (1988). Attraction of *Agrobacterium tumefaciens* C58C¹ towards sugars involves a highly sensitive chemotaxis system. *Journal of General Microbiology* **134**, 1427–1432.
- MILLER, J. H. (1972). *Experiments in Molecular Genetics*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- NESTER, E. W., GORDON, M. P., AMASINO, R. M. & YANOFSKY, M. (1984). Crown gall: a molecular and physiological analysis. *Annual Review of Plant Physiology* **35**, 387–413.
- OKKER, R. J. H., SPAINK, H., HILLE, J., VAN BRUSSEL, T. A. N., LUGTENBERG, B. & SCHILPEROORT, R. A. (1984). Plant inducible promoter of the *Agrobacterium tumefaciens* Ti-plasmid. *Nature, London* **312**, 564–566.
- PETERS, N. K., FROST, J. W. & LONG, S. R. (1986). A plant flavone, luteolin, induces expression of *Rhizobium meliloti* nodulation genes. *Science* **233**, 977–980.
- REDMOND, J. W., BATLEY, M., DJORDJEVIC, M. A., INNES, R. W., KUEMPEL, P. L. & ROLFE, B. G. (1986). Flavones induce expression of nodulation genes in *Rhizobium*. *Nature, London* **323**, 632–635.
- ROGOWSKY, P. M., CLOSE, T. J., CHIMERA, J. A., SHAW, J. J. & KADO, C. I. (1987). Regulation of the *vir* genes of *Agrobacterium tumefaciens* plasmid pTiC58. *Journal of Bacteriology* **169**, 5101–5112.
- ROSSEN, L., DAVIS, E. O. & JOHNSTON, A. W. B. (1987). Plant-induced expression of *Rhizobium* genes involved in host specificity and early stages of nodulation. *Trends in Biochemical Sciences* **12**, 430–433.
- SHAW, C. H., ASHBY, A. M. & WATSON, M. D. (1986). Plant tumour induction. *Nature, London* **324**, 415.
- SHAW, C. H., ASHBY, A. M., BROWN, A., ROYAL, C., LOAKE, G. J. & SHAW, C. H. (1988). *virA* and *G* are the Ti-plasmid functions required for chemotaxis of *Agrobacterium tumefaciens* towards acetosyringone. *Molecular Microbiology* **2**, 413–418.
- STACHEL, S. E. & ZAMBRYSKI, P. (1986). *virA* and *virG* control the plant-induced activation of the T-DNA transfer process of *A. tumefaciens*. *Cell* **46**, 325–333.
- STACHEL, S. E., MESSENS, E., VAN MONTAGU, M. & ZAMBRYSKI, P. (1985). Identification of the signal molecules produced by wounded plant cells that activate T-DNA transfer in *Agrobacterium tumefaciens*. *Nature, London* **318**, 624–629.
- STACHEL, S. E., NESTER, E. W. & ZAMBRYSKI, P. C. (1986). A plant cell factor induces *Agrobacterium tumefaciens vir* gene expression. *Proceedings of the National Academy of Sciences of the United States of America* **83**, 379–383.
- WINANS, S. C., EBERT, P. R., STACHEL, S. E., GORDON, M. P. & NESTER, E. W. (1986). A gene essential for *Agrobacterium* virulence is homologous to a family of positive regulatory loci. *Proceedings of the National Academy of Sciences of the United States of America* **83**, 8278–8282.
- ZAAAT, S. A. J., WIJFFELMAN, C. A., SPAINK, H. P., VAN BRUSSEL, A. A. N., OKKER, R. J. H. & LUGTENBERG, B. J. J. (1987). Induction of the *nodA* promoter of *Rhizobium leguminosarum* sym plasmid pRL1J1 by plant flavanones and flavones. *Journal of Bacteriology* **169**, 198–204.