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Chemotaxis, induced gene expression and competitiveness in the rhizosphere

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Abstract

Rhizobia are soil bacteria which symbiotically infect legume roots and generate nodules in which they fix atmospheric nitrogen for the plant in exchange for photosynthetically fixed carbon. A crucial aspect of signal exchange between these symbionts is the secretion of phenolic compounds by the host root which induce *nod*ulation gene expression in the bacteria. Stimulation of *nod* gene expression by host phenolics is required for nodule formation, is biochemically specific at 10^{-6} *M*, and is mediated by *nodD*. We and others have shown that rhizobia display chemotaxis to 10^{-9} *M* of the same phenolic compounds. Chemotaxis to inducer phenolics is selectively reduced or abolished by mutations in certain *nod* genes governing nodulation efficiency or host specificity. Conversely, mutations in rhizobia that affect general motility or chemotaxis have substantial effects on nodulation efficiency and competitiveness. These findings suggest that microbes entering the rhizosphere environment may utilize minor, non-nutrient components in root exudates as signals to guide their movement towards the root surface and elicit changes in gene expression appropriate to this environment.

Introduction

Chemically and physically, the rhizosphere is perhaps the most complex and changeable of all environments (Curl and Truelove, 1986). In this kind of environment, any microbe which is chemotactic, which can move to optimize its chemical inputs as things change, could have an important competitive edge. Unfortunately, there have been few studies to address the physiological ecology of microbial chemotaxis in the rhizosphere (*cf* Ames and Bergman, 1981; DeWeger *et al.*, 1987; El Haloui *et al.*, 1986; Griffin and Quail, 1968; Howie *et al.*, 1987; Hunter and Fahring, 1980; Madsen and Alexander, 1982; Scher *et al.*, 1988). A great deal has been learned in recent years about the cellular workings and machinery of chemotaxis in *E. coli* and Salmonella. Over 60 genes have been characterized, encoding signal detectors, transducers, wires, switches, motors, shafts, propellors, and system integrators (Koshland, 1981; McNab, 1987; Wolfe *et al.*, 1987). However, almost nothing is known about this machinery in any rhizobacterial species, and essentially nothing is known about the ecological role and functioning of this machinery in any bacteria, including *E. coli* (Chet and Mitchell, 1976; McNab, 1987). So, while chemotaxis is a common feature among soil bacteria (Bergey 1986), it demands a significant fraction of their total metabolic/genetic resources (McNab, 1987), and may be crucial to rhizosphere dynamics, this review describes an infant field, with all the dangers of error and oversimplification which that entails.

Presumably the adaptive value of chemotaxis involves movement towards or away from certain substances in the environment. But for bacteria that live in soil, what are the substances to seek and avoid? At what concentrations do they need

to be detected? What niches are occupied by chemotactic bacteria that cannot be occupied successfully by non-motile species? How important is chemotaxis to successful colonization of the rhizosphere? What substances from the root leak through an established surface microflora in high enough concentrations to elicit taxis? How are chemotactic responses to root substances integrated with changes in gene expression appropriate to the rhizosphere environment? Is there appreciable movement of bacteria on the root surface within the mucigel layer? What controls exist to regulate flagellar synthesis and activity levels in different soil and root environments? In our view, answers to such questions are fundamental to understanding the dynamics of microbial competition in the soil and rhizosphere.

Chemotaxis and the lifestyle of rhizobia

This review is concerned with recent studies on the role of chemotaxis in the symbiotic lifestyle of rhizobia. Rhizobia thrive in three very different environments: bulk soil, rhizosphere and host issue. They survive in fallow soils for many years, where they compete against diverse chemoorganotrophic soil bacteria and maintain population densities between 10^2 and 10^5 cells/g soil (Bowen and Rovira, 1976; Demezas and Bottomley, 1986; Rovira, 1961; Schmidt, 1979). Rhizobia appear to be excellent scavengers of organic compounds, and may depend largely on materials that escape utilization by faster-growing bacteria. For example, some rhizobia grow faster on 10^{-7} *M* succinate than certain strains of Enterobacter and Pseudomonas, although not as rapidly as these strains at higher concentrations (Humbeck *et al.*, 1985). The scavenging ability of rhizobia is also indicated by the fact that they (and other soil bacteria) can survive for long periods, and multiply extensively in distilled water (Crist *et al.*, 1984; Humbeck *et al.*, 1985). While it is known that rhizobia can move chemotactically through samples of autoclaved soils (Soby and Bergman, 1983) and move to micromolar concentrations of various organic and aromatic acids (Parke *et al.*, 1985), the role of chemotaxis in long-term survival in the soil does not appear to have been investigated for rhizobia or any other soil bacterium.

Rhizobia also are successful competitors in the rhizosphere of both host and non-host plants, achieving population densities 5 to 100 times higher than in bulk soil (*e.g.* Mowad *et al.*, 1984; Schmidt, 1979). They have a slower growth rate than dominant species like Pseudomonas, but nonetheless can establish and maintain themselves at about 2% to 20% of the total number of bacteria in the rhizosphere of their host (Mowad *et al.*, 1984). Recent studies with Pseudomonas (DeWeger *et al.*, 1987; Howie *et al.*, 1987) indicate that chemotaxis can be either very important or unimportant in rhizosphere colonization, depending on circumstances. The importance of chemotaxis to rhizosphere colonization by rhizobia has never been examined.

Although very respectable soil saprophytes and rhizosphere colonizers, rhizobia have received most attention for their ability to establish intimate symbiotic associations with roots of legumes (Bauer, 1981; Djordjevic *et al.*, 1987; Rolfe and Gresshoff, 1988). During the infection process, the first visible events are movement of rhizobia to the root and their attachment to the surface (Gulash *et al.*, 1984; Pueppke, 1984). Firm attachment is frequently polar (Bohlool and Schmidt, 1976) and may involve pili (Vesper and Bauer, 1986). Shortly after inoculation, rhizobia on or near the root surface induce at least two crucial responses in the host: the curling of growing root hairs and the localized dedifferentiation of root cells in the root cortex. The elicitation of these host responses depends on the expression of certain *nod*ulation genes (*nodABC*) in the bacterium. The expression of these *nod* genes is elicited, in turn, by exposure of the bacteria to specific phenolic compounds secreted by the host root. Induction of the *nodABC* genes is known to involve a regulatory gene, *nodD* (Mulligan and Long, 1985; Rossen *et al.*, 1986; Spanik *et al.*, 1987).

In most of the host legumes studied, only young root hairs can be induced to curl sufficiently to entrap Rhizobium cells in a pocket of host cell walls. In soybean and alfalfa, such entrapment is complete within 8 to 12 h after contact and seems to be required for subsequent penetration and infection (Dudley *et al.*, 1987;

Turgeon and Bauer, 1985). Diffusible substances from rhizobia on (or near) the root surface rapidly induce the host-specific formation of many localized centers of cell division in the outer cortex. Further rounds of cell division lead to the creation of new meristems. The bacteria-induced meristems differentiate to form nodules in which the rhizobia, packaged densely within host cells, convert atmospheric N_2 into NH_3 and receive fixed carbon from the host plant.

Individual root cells are susceptible to symbiotic induction of root hair curling and cortical cell division for a period of only 6 to 12 h (Bhuvaneswari *et al.*, 1981; Calvert *et al.*, 1984). The acquisition and loss of susceptibility to rhizobia appears to be governed by acropetal root development, restricting new infections to the zone of emergent root hairs near the root tip. Nodule initiation is therefore a very dynamic process, one which a bacterial cell can elicit only if it gets to the right place on the surface, and if it does so in time to respond to host phenolics that induce *nodABC* expression and produce enough of the *nodABC*-related signal substance(s) to provoke the necessary developmental changes in nearby host cells. These constraints suggest that nodule initiation might depend significantly on rapid, directed movement of bacteria to, and on, the root surface.

The role of chemotaxis in nodule formation by rhizobia

Chemotaxis appears to have an important role in nodule initiation. Early work established that non-motile mutants of rhizobia were able to infect and nodulate their hosts (Ames *et al.*, 1980; Ames and Bergman, 1981; Hunter and Fahring, 1980; Napoli and Albersheim, 1980), demonstrating that chemotaxis is not required for nodulation. However, non-motile mutants have been shown to be at a serious competitive disadvantage (Ames and Bergman, 1981; Caetano-Anollés *et al.*, 1988b; El Haloui *et al.*, 1986; Hunter and Fahring, 1980). When alfalfa roots were inoculated with an equal mixture of wild-type and non-motile mutant cells, wild-type *R. meliloti* cells generated 2 to 6 times more nodules than the mutant. Similarly, wild-type *B. japonicum* 110 formed about 6 times more nodules on soybean than equal numbers of a mutant with reduced motility. Based on the log-linear dose-response behavior of strain 110, the inoculum mixture would have to contain approximately 100 times more cells of the mutant than the wild-type to obtain equal nodule occupancy.

Motility and chemotaxis in rhizobia have not been well characterized. The only genetic analysis to date is in *R. meliloti* (Ames *et al.*, 1980; Bergman *et al.*, 1988; Ziegler *et al.*, 1987). Some work has been done to characterize the components of flagellar complexes or the dynamics of movement during chemotaxis in rhizobia (*cf.* DeLey and Rassel, 1965; Gotz and Schmitt, 1987; Gotz *et al.*, 1982; Krupski *et al.*, 1985; Schleicher and Bergman, 1981; Schmitt *et al.*, 1974). Others have assayed chemotaxis of rhizobia towards root exudates, or towards sugar and amino acid components of root exudates (Burg *et al.*, 1982; Currier and Strobel, 1976, 1981; Gaworzewska and Carlisle, 1982; Gitte *et al.*, 1978; Kandsamy and Prasad, 1979). Videomicroscopy has revealed that chemotaxis to the host root might be rather more focused, specific and interesting than previously imagined: chemotactic swarms of *R. meliloti* cells were observed to form at certain highly localized sites in the infectible zone of alfalfa roots and new swarms developed at the same sites after rinsing (Gulash *et al.*, 1984).

Our initial studies (Caetano-Anollés *et al.*, 1988b) sought to determine when and how chemotaxis contributed to nodulation. It appears that chemotaxis makes important contributions to several stages of the infection process, not just a single stage. Even after flooding roots with mutant/parent mixtures, the wild-type occupied 3 to 10 times more of the first-formed nodules than non-chemotactic mutants. These results demonstrated for the first time that chemotaxis enhances steps in nodule initiation during the first few hours after contact. Dose-response studies showed that 10 to 50 times more cells of the non-chemotactic mutants would have to be added to initiate the same number of nodules as the wild-type. The wild-type strain attached to the root surface in greater numbers than the mutants, but these differences accounted for less than half of its higher nodulation efficiency

(Caetano-Anollés and Bauer, 1988) suggesting that chemotaxis must confer other symbiotic advantages. Infections by non-motile mutants were somewhat slower to develop after initiation, so that the emergence of nodules formed by non-chemotactic mutants was delayed about one day relative to wild-type nodules.

In unpublished studies, we examined the ability of *R. meliloti* cells to move and multiply on the root surface by placing a 10 nanoliter droplet containing just 1 to 10 bacteria onto the surface of alfalfa roots maintained in sterile growth pouches. Both nodules and bacteria appeared in younger regions of the root, several cm below the point of inoculation. The bacteria multiplied extensively on the root surface in more mature regions of the root. In general, however, the rate of bacterial spread and multiplication were not sufficient to maintain a substantial population density of *R. meliloti* in the zone of emerging root hairs, the only zone susceptible to infection in alfalfa (Bhuvaneswari *et al.*, 1981). In agriculture, it may be the failure of inoculum strains to maintain an adequate presence in the infectible regions of the root, near the growing tips, that most limits their effectiveness in the field.

Using spot-inoculation techniques, we found that the wild-type bacteria formed 1.5 to 5 times more nodules in the initially susceptible region of the root than non-motile mutants. Since the bacteria were placed directly onto the root and remained there, enhanced nodulation by the wild-type must have been due to movement *on* the surface rather than movement to the root. This suggests that chemotaxis may enable rhizobia to reach microsites more suitable for infection after initial contact with the root. Such movement and its regulation could have a major bearing on symbiotic competition between strains of rhizobia.

In unpublished work, we have enriched non-mutagenized cultures of *Bradyrhizobium japonicum* I-110 through 7 cycles for cells from the outer edge of swarm colonies. At least three independent isolates were obtained which formed swarm colonies that increased in diameter 2–3 times faster than the wild-type on soft agar swarm plates. The basis for the enhanced chemotaxis has not yet been determined. Since only *some* of the isolates we obtained through this serial enrichment showed enhanced chemotaxis, it appears that the more active isolates are in fact spontaneous mutants. One of these 'hypermotile' mutants was recently tested for nodulation ability on soybean and was found to be significantly more efficient in nodule initiation than the parent. Dose-response studies indicate that 5 to 10 times as many wild-type bacteria would have to be added to achieve the same level of nodulation as this mutant. Enhanced nodulation by the hypermotile mutant is evidence that chemotaxis contributes to the efficiency of nodule initiation, as is reduced nodulation by the non-motile mutant.

Chemotaxis to phenolic compounds from the host root

Recent studies in Nester's and Shaw's labs revealed that *A. tumefaciens* was chemically attracted to several of the same host root phenolics that are required for induction of the *vir*ulence genes in this bacterium (Ashby *et al.*, 1987, 1988; Parke *et al.*, 1987; Shaw *et al.*, 1988a,b). Acetosyringone (2,4,6-trimethoxy phenol) is selectively produced in wounded tissue, and wounds are the site of *A. tumefaciens* infection. Acetosyringone was found to elicit maximum chemotaxis at concentrations of about 10^{-7} *M*, which is about 100-fold lower than the concentration required for maximal induction of the *vir* genes. From these results, it appears that the pathogen is able to detect very low concentrations of this wound-specific compound, swim towards it by chemotaxis, and then turn on its *vir*ulence genes when it gets close to the wound site.

Just as *A. tumefaciens* responds chemotactically towards phenolic inducers of *vir* gene expression, we have shown that *R. meliloti* responds chemotactically to host flavonoids that act as inducers of *nod* gene expression. An earlier study by Peters *et al.* (1986) identified the flavone luteolin from alfalfa seeds as a potent inducer of *nod* genes in *R. meliloti*, active at concentrations of about 10^{-6} to 10^{-7} M. We found that luteolin elicits half-maximal chemotactic responses in *R. meliloti* at concentrations of 10^{-9} M or less (Caetano-Anollés *et*

al., 1988a). The addition of naringenin, a close structural relative of luteolin, blocked chemotaxis of *R. meliloti* to luteolin, just as it specifically blocked *nodD1*-mediated induction of the *nodABC* genes by luteolin (Peters and Long, 1988). This suggests that *nod* gene induction and chemotaxis elicited by luteolin may share a common receptor or other components of their signal transduction pathways. The addition of apigenin, another close structural relative of luteolin, also had parallel effects on both induction of *nod* genes and chemotaxis. At the genetic level, we observed that *nodD1*::Tn5 mutants of *R. meliloti* failed to respond to luteolin as a chemoattractant, whereas these same mutants responded normally to amino acids and other common attractants, indicating that the *nodD1* gene may have some role in chemotactic responses to luteolin (Caetano-Anollés *et al.*, 1988a). Similarly, Shaw *et al.* (1988a) reported that the *virA* and *virG* genes were required for chemotaxis of *A. tumefaciens* to acetosyringone. Thus, there is both biochemical and genetic evidence to indicate that gene induction by host phenolics and chemotaxis towards these compounds are highly specific and interrelated processes. Similar evidence of chemotaxis towards *nod* gene-inducing host phenolics has been obtained with other rhizobia, including *R. leguminosarum* bv. *viciea* (Armitage *et al.*, 1988) and *R. leguminosarum* bv. *phaseoli* (Aguilar *et al.*, 1988).

Chemotaxis towards host phenolics is not always linked to induction of new gene expression. In *R. leguminosarum* and *A. tumefaciens*, it seems that some host phenolics serve only as attractants or only as inducers of new gene expression, but not both. And different strains of a symbiont or pathogen may prove to differ in their responses to a particular phenolic compound, reflecting different coevolutionary histories and regulatory strategies. Nonetheless, it seems likely from present evidence that many microorganisms, pathogenic, symbiotic and saprophytic, may rely on specific members of their host's array of secreted phenolics to trigger both taxis and new gene expression appropriate to the rhizosphere environment.

Two points of caution deserve mention in this regard. First, it is important to recognize that the flavonoids or other phenolics that a host plant secretes into the rhizosphere may be quite different from those sequested in the seed or root tissues. Furthermore, the spectrum of phenolics in root exudates may change dramatically with plant age, genotype, growth environment and exposure to microorganisms. The recent studies of Maxwell *et al.* (1989) and Hartwig *et al.* (1989) provide particularly noteworthy examples of analysis, but there is a great deal still to be learned about the secretion of host phenolics before their biological role in plant-microbe interactions can be properly assessed and usefully manipulated.

Second, it is our experience, shared by others, that the chemotactic responses of bacteria to host phenolics are weak and variable, at least under laboratory culture and assay conditions. Chemotactic responses to acetosyringone and the various *nod* gene inducing flavonoids typically range between 2 to 5 times higher than background, in contrast to the 10- to 100-fold responses elicited by organic and amino acids. The weaker chemotaxis of *Rhizobiaceae* towards host phenolics may be due to the much lower concentrations of phenolics which elicit maximal responses. Peak responses to the phenolics usually occur at concentrations about 10^4-fold lower than those eliciting maximal responses to amino acids or other common nutrients. It may be that a gradient of attractant ranging between 10^{-8} M and 10^{-9} M simply cannot provide enough of a differential in attractant molecules bound to bacterial transducer proteins to generate a strongly biased random walk, just as it's hard to locate where smoke is coming from if it is so dilute that you can barely detect it. Alternatively, it may be that chemotactic responses to the phenolics are suppressed in bacteria cultured under laboratory conditions. In this regard, Peters and Long (1988) observed that *R. meliloti* cultured on a rich medium was about 20-fold less responsive to luteolin induction of *nod* genes than when cultured on a minimal medium. With regard to variability in chemotaxis towards host phenolics, we note that wild-type *R. meliloti* RCR2011 has twice spontaneously lost its responsiveness to luteolin in the past three years, forcing us to use isolates from other labs. And while our earlier studies indicated that chemotactic responses to luteolin were abolished by mutations in the

nodA and *nodC* genes as well as by mutations in the *nodD1* gene (Caetano-Anollés *et al.*, 1988a), the chemotactic responses of these *nod* mutants to luteolin have been inconsistent in more recent experiments, usually negative but occasionally positive. Strains carrying sequences that complement the mutations have shown similarly inconsistent chemotaxis towards luteolin. These results suggest that chemotaxis to luteolin is an unstable trait in *R. meliloti*, at least in strain RCR2011, and may not be directly dependent on the expression of the *nod* genes.

Bergman *et al.* (1988) recently described a mutant of *R. meliloti* which is defective in general chemotaxis towards common nutrients of soft agar plates, but is perfectly capable of forming chemotactic swarms at specific sites on the host root surface. These results strongly imply that *R. meliloti* has two relatively independent pathways of chemotactic response: one set of responses to common nutrients and another set of responses to substances from the host root, perhaps non-metabolized compounds like luteolin. This concept of a dual chemotaxis pathway, as proposed by Bergman *et al.*, suggests a more general possibility: that rhizosphere bacteria may rely on certain poorly metabolized host substances as important sources of information about the proximity and genotype of a nearby root or seed. If this idea is correct, as the available evidence indicates, then the molecular details of signal transduction and response to such compounds should be of considerable importance to the competitiveness of bacteria in colonizing the rhizosphere.

Conclusions and directions

In summary, there are now several good reasons to believe that chemotaxis is important to rhizosphere interactions and symbiotic infection by rhizobia: non-motile mutants are less competitive in nodule initiation and occupancy; they are also slower in infection development; hypermotile mutants have improved ability to initiate nodules; rhizobia form chemotactic swarms at local sites on host roots; they respond chemotactically to host substances that induce *nod* gene expression; and expression of several *nod* genes is required for chemotaxis to these compounds.

These results suggest that further studies on the role of chemotaxis in rhizosphere colonization and host interactions are warranted. Studies are needed to establish when, how, and how much, chemotaxis contributes to the growth and survival of bacteria in soil or rhizosphere environments. These questions would seem to be of basic importance to soil microbiology, particularly in regards to the dynamics of competition for energy sources. Initial studies are needed to provide at least some measure of the overall costs and benefits associated with chemotaxis in soil rhizosphere environments. The isolation and characterization of defined mutants with altered chemotaxis can provide effective tools for assessing such costs and benefits. Information from such experiments may be useful in devising ways to enhance the effectiveness of genetically engineered inoculum bacteria or to limit their spread and long term survival in the soil.

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