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Agrobacterium tumefaciens Mutants Deficient in Chemotaxis to Root Exudates

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A directional swarm assay on semisolid agar was used to measure chemotaxis in *Agrobacterium tumefaciens*. Several *A. tumefaciens* strains, including two lacking Ti plasmids, were attracted to root exudates from isolated root cap cells or from excised root tips of pea. Only 300 sloughed pea cells were required to elicit chemotaxis, and the relative distance moved by the bacteria increased with increasing numbers of plant cells up to approximately 4,000 cells. Tn*5* mutagenesis was used to generate strains that did not respond chemotactically to root exudates. The mutants included several categories: nonmotile mutants, slow-migrating mutants, and mutants with normal motility that were not attracted to root exudates. In addition, one Tn*5* mutant was attracted to excised root tips but not to isolated root cap cells from pea. A quantitative root cap cell binding assay was used to demonstrate that all of the isolated mutants exhibit wild-type binding levels.

Additional keywords: crown gall, motility, rhizosphere colonization.

Agrobacterium tumefaciens is a soil-borne bacterial pathogen that causes a tumorigenic disease called crown gall on most dicots and some gymnosperms and monocots (DeCleene 1985). It infects through wounds, primarily in roots and stems. Various *Agrobacterium* species and biotypes have been found in diverse terrestrial environments inhabited by both host and nonhost plant species, and the bacteria can invade cultivated soils and cause epidemics (Bouzar and Moore 1987). However, despite the rapid progress that has been made in elucidating the molecular biology of crown gall pathogenesis (Stachel and Zambryski 1986), little is known about how *A. tumefaciens* recognizes host plants under natural conditions and becomes established in sufficiently high concentrations in the rhizosphere and on the plant to initiate infection.

One possibility is that the bacteria are attracted to molecules exuded or secreted by plant roots (Ashby *et al.* 1987). Chemotaxis in bacteria involves the directed movement of motile cells in response to a gradient of attractant or repellant (Adler 1966). The phenomenon in enteric bacteria has been used extensively as a model system for investigating the molecular bases of how organisms detect and process sensory information (Adler 1975; Parkinson 1981). Chemotaxis has also been observed in several soil-borne or plant pathogenic bacteria, including *Erwinia amylovora* (Raymundo and Ries 1980), *Pseudomonas aeruginosa* (Craven and Montie 1985; Moench and Konetzka 1978), *P. lachrymans* (Chet *et al.* 1973; Chet and Mitchell 1976), *P. phaseolicola* (Panopoulos and Schroth 1974), several *Rhizobium* species (Ames and Bergman 1981; Ames *et al.* 1980; Currier and Strobel 1976; Gaworzewska and Carlile 1982; Gitte *et al.* 1978; Hunter and Fahring 1980), *Xanthomonas oryzae* (Feng and Kuo 1975), *Azospirillum lipoferum* (Barak *et al.* 1983; Heinrich and Hess 1985), and *A. tumefaciens* (Ashby *et al.* 1987; Beiderbeck and Hohl 1979; Parke *et al.* 1987).

Recently, Ashby *et al.* (1987) reported that a Ti plasmid encoded function can mediate chemotaxis in *A. tumefaciens* strain C58 toward acetosyringone (AS), an inducer of *Agrobacterium* virulence (*vir*) genes. Maximum attraction occurs at 10^{-7} M, a concentration that is two orders of magnitude lower than that required for *vir* gene induction. The authors proposed that chemotaxis toward AS may constitute the first step in specific recognition between *A. tumefaciens* and host plants in soil. However, the specific function of AS as a chemotaxis signal has been disputed by Parke *et al.* (1987), who reported that *A. tumefaciens* strain A348 is not attracted to AS, that maximal attraction to other monocyclic phenolic compounds occurs at much higher concentrations than reported by Ashby *et al.* (1987), and that chemotaxis is Ti plasmid independent.

Even though the specific function of AS in chemotaxis is controversial, the results do not negate the potential significance of chemotaxis in the biology of crown gall pathogenesis. The objective of our work was to establish an assay to detect migration of *A. tumefaciens* toward plants or isolated plant cells and to use the assay to isolate mutants deficient in chemotaxis toward such complex substrates.

MATERIALS AND METHODS

Bacteria. *A. tumefaciens* cultures derived from single colony isolates were grown overnight on yeast extract-mannitol (YEM) agar and then suspended in water. Concentrations were estimated turbidimetrically and confirmed by dilution plating onto nutrient medium. Stock cultures were maintained in 50% glycerol:YEM (Bhuvanesari *et al.* 1977) at −80° C.

Chemotaxis assays. Seeds of *Pisum sativum* 'Little Marvel' were surface sterilized by immersion for 5 min in 95% ethanol followed by 5 min in 0.05% sodium hypochloride and then rinsed four times in 200 ml of sterile distilled water. Seedlings were germinated at 27° C for 3 days on 0.7% water agar overlaid with filter paper. One or two root tips (3 mm in length) were excised and placed at the edge of a semisolid swarm plate: a petri plate (5 cm in diameter) containing approximately 5 ml of water, YEM, or 10 mM K_2PO_4 solidified with 0.2% agar. A 10-μl droplet

containing 10^5, 10^6, or 10^7 bacteria (10^7, 10^8, or 10^9 colony-forming units per milliliter) was placed into the center of the plate. After 16 hr the ratio of the distance that the bacterial swarm moved toward roots and away from roots, the chemotaxis ratio, was measured. Alternatively, root cap cells from six seedlings were isolated nondestructively into 100 μl of water as described previously (Hawes and Pueppke 1987). A 10-μl droplet of the root cap cell suspension containing approximately 2,000 cells (Hawes and Pueppke 1986) was placed at the edge of a swarm plate, and the chemotaxis ratio was measured as described above. In the dosage-response assay, root cap cell numbers were estimated with a hemacytometer and were confirmed by direct counts. Suspensions of *Chlorella* isolate NC64A were grown in MBBM medium (Van Etten *et al.* 1983), and were concentrated to a density of 10^7 per milliliter. A 10-μl sample of the cells, either washed twice in water or taken directly from culture medium, was added to the edge of a plate of water agar as described above. To assay chemotaxis to simple substrates, a 10-μl droplet of an amino acid or sugar solution (10 mM) was placed at the edge of semisolid agar plates, and bacterial movement was measured.

Isolation and characterization of Tn*5* mutants. Tn*5* mutants were generated by mating *A. tumefaciens* strain A348 with *E. coli* strain 1830(pJB4JI) by established procedures (Garfinkel and Nester 1980). Each kanamycin-resistant mutant was screened for chemotaxis both to root tips and to isolated root cap cells by the assay described above. Mutants that did not move from the central point of inoculation, or those that moved but did not migrate toward the source of attractant, were selected for further analysis. To classify mutant phenotypes, all putative chemotaxis mutants were tested by all of the following assays: 1) Production of β-ketolactose, which is diagnostic for *A. tumefaciens*, biotype 1, was tested by the method of Bernaerts and DeLey (1963). 2) Motility was tested by measuring the diameter of bacterial colonies 16 hr after placing a 10-μl drop of inoculum at the center of plates of nutrient semisolid swarm agar and by microscopic observation of swimming behavior. 3) Chemotaxis to sugars and amino acids was measured as described above. 4) Binding was measured by counting the number of bacteria that attach to isolated root cap cells as described previously (Hawes and Pueppke 1987). 5) The presence of Tn*5* was confirmed by slot blot filter hybridization. A slot manifold device and vacuum were used to deposit approximately equal molar amounts of each DNA onto Gene Screen (New England Nuclear). Thus, 5 μg of DNA from each mutant or strain, 6.4 ng of pKC7 (a plasmid that contains the Tn*5* kanamycin-resistance gene) (Rao and Rogers 1979), and 50 ng of lambda DNA were diluted in 0.2 ml of water and deposited on the filter. DNA was bound to the filter by successive 1-min immersions in 0.4 N NaOH and 25 mM sodium phosphate buffer, pH 6.5, followed by shortwave ultraviolet irradiation from a distance of 15 cm for 3 min. Prehybridization was in a solution containing 50 mM Tris-HCl, pH 7.5, 1 M NaCl, 50% deionized formamide, 1% sodium dodecyl sulfate (SDS), 10% dextran sulfate, 0.1% sodium pyrophosphate, and 0.2% each of bovine serum albumin, polyvinylpyrrolidone (M_r 40,000), and Ficoll (M_r 400,000) at 35° C for at least 1 hr. Hybridization was in the same solution containing approximately 10^6 cpm of P^{32}-labeled probe. The probe was made from pKC7 by random primer labeling (Feinberg and Vogelstein 1983) after disruption of the supercoiled conformation by digestion with *Hind*III and *Xho*I. After hybridization for 16 hr, the filter was washed twice in 0.01 SSC (1.5 mM NaCl, 0.15 mM sodium citrate) containing 0.1% SDS and 0.5% saturated sodium pyrophosphate at 25° C for 30 min. Autoradiography was by exposure to Kodak XAR film without intensifying screens.

RESULTS

Development of an assay for chemotaxis toward root exudates. A modified swarm assay based on the work of Adler (1966, 1975) was used to measure chemotaxis. After an overnight incubation on semisolid medium, *A. tumefaciens* strain A348 exhibited positive chemotaxis toward isolated pea root cap cells (Fig. 1). The directional swarming response did not appear to be affected by the presence of an energy source (YEM), phosphate buffer, or EDTA. Subsequent assays were therefore conducted on the simplest medium, water agar. It was possible to measure the response based on a chemotaxis ratio, or the ratio of the distance that the bacterial swarm moved toward the source of attractant to the distance moved away from the attractant. The number of bacteria included in the assay did not affect the distance that the swarm moved toward the attractant. However, the response was much more distinct with higher concentrations of bacteria. At concentrations below 10^8 per milliliter (a total of 10^6 bacteria per assay), the swarm was barely visible, if at all, even though bacteria could be recovered from the agar at a distance from the center. Chemotaxis to suspensions of isolated root cap cells was influenced by the number of cells. As few as 300 root cap cells were required to induce visible chemotactic movement, and chemotaxis ratios increased with increasing numbers of isolated root cap cells (Table 1). The response apparently saturates at concentrations greater than approximately 4,000 plant cells. *A. tumefaciens* strain A348 also moved to root tips of maize, bean, and cotton, and to root cap cells of maize (Table 2). In contrast, isolated root cap cells from bean and cotton were inactive as attractants. *A. tumefaciens* was not attracted to suspensions of isolated *Chlorella* cells.

There was no measurable difference in chemotaxis among several wild-type strains, or between the Ti plasmid plus and minus strains ACH5 and ACH5C3, and 15955 and

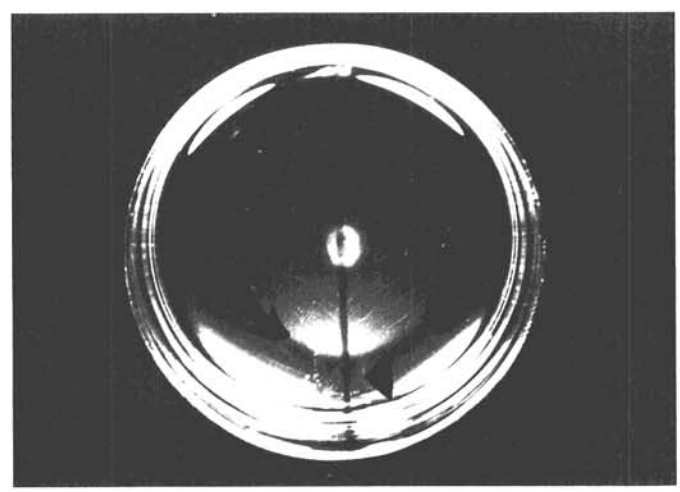

Fig. 1. Directional swarm assay. Chemotaxis of *A. tumefaciens* on semisolid water agar is expressed as an expanding disc of bacteria (arrow) that moves from a central point of inoculation only in the direction of a 10-μl droplet of isolated root cap cells suspended in water (triangle denotes site of cells, which are not visible in the figure). The chemotaxis ratio is the ratio of the distance moved toward the attractant to the distance moved in the opposite direction.

NT1(15955), respectively (Table 3). We chose to select mutants in strain A348, which contains pTiA6 in the C58 chromosomal background and which has been evaluated in previous chemotaxis studies (Parke *et al.* 1987).

Chemotaxis of strain A348 toward purified chemicals. In the swarm assay, *A. tumefaciens* was attracted to the following amino acids and sugars: threonine, glutamine, proline, histidine, asparagine, alanine, glucose, fructose, arabinose, sucrose, xylose, and mannose. Phenylalanine, isoleucine, valine, methionine, raffinose, galactose, and rhamnose were not attractive to the bacteria. Acetosyringone at concentrations from 10^{-2} to 10^{-12} M also failed to elicit chemotactic movement in our assay.

Isolation and analysis of Tn*5* mutants. Of 1,600 Tn*5* mutants screened, 11 failed to exhibit wild-type chemotaxis toward root tips or root cap cells. Of the 11, seven mutants (C563, C645, C710, C1530, C1669, C1786, and C294) were defective in motility. Five of the motility mutants failed to exhibit any movement under microscopic observation or to swarm on nutrient swarm plates (Fig. 2D) or in chemotaxis assays (Fig. 3A). One mutant, C1786, moved more slowly than the parent in swarm assays (Fig. 2C) and in chemotaxis assays (Fig. 3C), but chemotaxis ratios for movement toward root cap cells and root tips were equal to those of the parent (Table 4). C1786 also was attracted to all of the amino acids that attract A348, but failed to move to any sugars. C294 moved slowly on swarm agar (Fig. 2C) and did not show differential movement toward root cap cells and root tips. It was attracted to all amino acids that attract A348, but only two sugars, arabinose and sucrose, that attract the parent. Four mutants exhibited wild-type movement on nutrient swarm plates, but were deficient in chemotaxis (Fig. 3B). Three of the mutants were not attracted toward root cap cells or root tips. Of those, C503 exhibited chemotaxis to sucrose, glutamine, histidine, and asparagine. The other two failed to move toward any tested substrates. Finally, one mutant with wild-type motility, C586, was attracted to root tips but not to root cap cells.

All of the mutants isolated to date exhibit wild-type levels of binding to isolated root cap cells; in two independent experiments, values for parent strain A348 and for all mutants ranged from 11 ± 1 to 12 ± 2 bound bacteria per cell perimeter. Three Tn*5* mutants that exhibit wild-type chemotaxis (18, 1718, and 3057) were included with the parent strain as positive controls in most tests. All motility, chemotaxis, and positive control Tn*5* mutants were positive for β-ketolactose production, and DNA from all of the mutants hybridized to a probe derived from the Tn*5* kanamycin-resistance gene.

Table 1. Influence of root cap cell concentration on relative movement of *Agrobacterium tumefaciens* in swarm agar chemotaxis assay

Number of root cap cells[a]	Chemotaxis ratio[b]
6,099	*1.69 ± 0.19
2,570	*1.71 ± 0.13
1,016	*1.56 ± 0.13
622	*1.47 ± 0.06
318	*1.21 ± 0.08
211	1.10 ± 0.02
61	0.94 ± 0.05
11	1.00 ± 0.09

[a] Root cap cell numbers were estimated using a hemacytometer and were serially diluted; numbers in dilutions were confirmed by direct counts. Strain A348 (10 μl at 10^8/ml) was added to the center of the plate, and the chemotaxis ratio was measured after 16 hr. Plant cell viability was 90–95% for the duration of the assay.
[b] Values are means from five replicate plates in duplicate assays. *, Values are significantly different from 1.0 at 0.01% level.

Table 2. Chemotactic responses of *A. tumefaciens* strain A348 to excised root tips and isolated root cap cells of different plants

	Chemotaxis ratio[a]	
Plant species	Root cap cells	Root tips
Pea	*1.67 + 0.12	*1.59 + 0.2
Corn	*1.74 + 0.17	*1.50 + 0.3
Pinto bean	1.00 + 0.16	*1.58 + 0.4
Tepary bean	1.00 + 0.07	*1.43 + 0.2
Cotton	1.00 + 0.04	*1.69 + 0.2

[a] All values are means from five replicate samples in two independent experiments. Approximately 3,000 root cap cells (estimated by direct counts) from each species were used in the root cap cell assay. (Each pea, bean, or corn root yields 2,000–4,000 cells; cotton seedlings yield 4,000–10,000 cells per root.) Three segments, 3–4 mm in length, were used in each excised root tip assay. *, Values are significantly different from 1.0 at the 0.01% level.

Table 3. Chemotaxis of *Agrobacterium* strains

		Chemotaxis ratio[b]		
Strain	Virulence on pea[a]	Root cap cells	Root tips	Source
A. tumefaciens				
A348	+	1.6	1.7	E. W. Nester
A723	+	1.7	1.6	E. W. Nester
C58	+	1.6	1.6	E. W. Nester
B6	+	1.5	1.6	A. Matthysse
Chryl	+	1.7	1.6	R. Stall
T37	+	1.6	1.6	E. W. Nester
15955	+	1.6	1.5	W. Gurley
ACH5	+	1.6	1.6	J. A. Lippincott
ACH5C3	−	1.6	1.5	J. A. Lippincott
A281	+	1.7	1.6	L. Owens
A. rhizogenes				
R1000	+	1.7	1.6	H. Flores

[a] Isolates were considered virulent if at least one of 10 seedlings developed tumors within 3 wk after inoculation; in most cases, all plants developed tumors.
[b] The chemotaxis ratio was measured as described in Materials and Methods. Values represent means of at least five replicate assays.

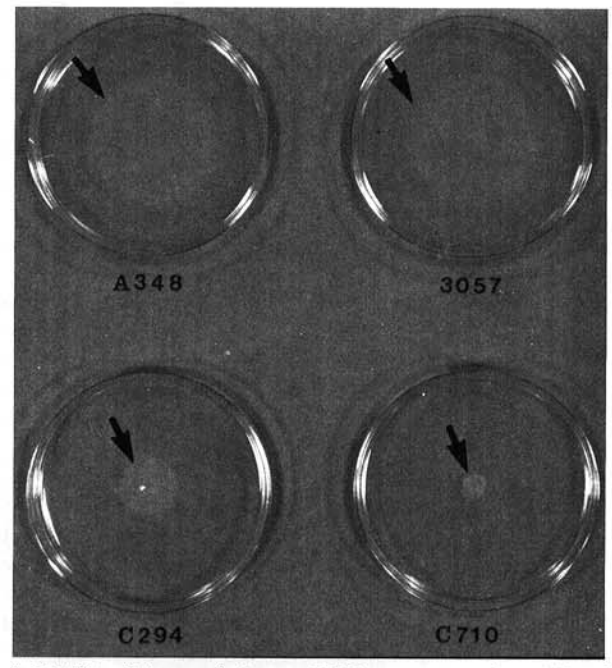

Fig. 2. Motility of *A. tumefaciens* on YEM swarm agar plates 16 hr after adding a 10-μl droplet of bacteria (10^8 per milliliter) to the center of the plate. Samples include parent strain A348, positive control Tn*5* mutant 3057, slow-migrating mutant C294, and nonmotile mutant C710. Arrows denote the leading edge of the swarm.

DISCUSSION

We have established an assay to measure migration of bacteria toward isolated root cells and tissue and have used the assay to evaluate chemotaxis properties of *A. tumefaciens* and to identify mutants by insertion of Tn*5*. We chose to study chemotaxis of *A. tumefaciens* to root exudates that are as nearly as possible in the form in which they occur when released from roots in nature. The root cap produces the bulk of the largely polysaccharide exudates that are secreted by plant roots. The use of isolated root cap cells provides a way to distinguish between chemotaxis to products that are naturally present in the rhizosphere of healthy plants and chemotaxis to exudates released from wounded tissue. Motile *A. tumefaciens* strains, with and without Ti plasmids, were strongly attracted both to root cap cells and to excised pea root tips. The bacteria also were attracted to both sources from maize. Attraction to root cap cells was dosage dependent, with a positive response to as few as 300 cells, or approximately 10% of the number that slough from a single root daily (Hawes and Pueppke 1986). That there is some specificity to the response is indicated by the fact that *A. tumefaciens* is not attracted to

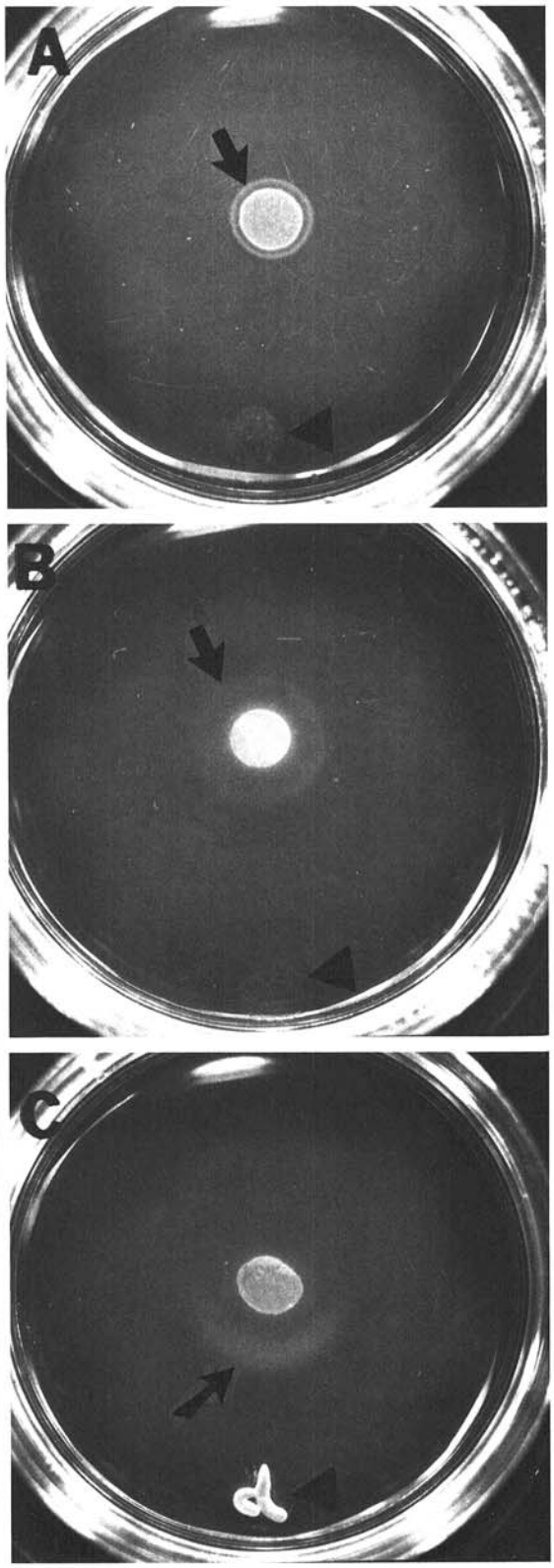

Fig. 3. Phenotypes of motility and chemotaxis mutants in chemotaxis assays. **A,** Nonmotile mutant C710; **B,** motile but nonchemotactic mutant C461; **C,** slow-migrating mutant C294. Arrows denote the leading edge of the bacterial swarm, and triangles denote the site of a droplet of isolated root cap cells **(A,B)** or excised roots tips **(C)**.

Table 4. Characteristics of nonchemotactic mutants[a]

Strain or mutant	Motility	Chemotaxis to: Cells	Root tips	Sugars	Amino acids
Parent					
A348	7.5 ± 1 mm	1.6 (11/7)	1.6 (12/7.5)	+	+
Positive Tn*5* control mutants					
18	5.5 ± 1	1.6 (10/6)	1.6 (9/5.5)	+	+
1718	7.5 ± 1	1.6 (11/7)	1.7 (12/7)	+	+
3057	7.0 ± 1	1.6 (11/7)	1.7 (12/7)	+	+
Chemotaxis mutants					
C461	6.0 ± 1	1.0 (3/3)	1.0 (3/3)	−	−
C503	6.5 ± 1	1.0 (3/3)	1.0 (3/3)	+[b]	+[c]
C531	7.0 ± 1	1.0 (3/3)	1.0 (3/3)	−	−
C586	6.0 ± 1	1.0 (4/4)	1.8 (7/4)	−	−
Slow-migrating mutants					
C1786	3.0 ± 1	1.6 (5/3)	1.6 (5/3)	−	+[d]
C294	2.0 ± 1	1.0 (6/6)	1.0 (6/6)	+[e]	+[f]
Nonmotile mutants					
C563	0	1.0 (3/3)	1.0 (3/3)	−	−
C645	0	1.0 (3/3)	1.0 (3/3)	−	−
C710	0	1.0 (3/3)	1.0 (3/3)	−	−
C1530	0	1.0 (3/3)	1.0 (3/3)	−	−
C1669	0	1.0	1.0	−	−

[a] Motility and chemotaxis assays are described in Materials and Methods. Motility is expressed as the radius of the distance (in mm) moved by the bacterial swarm from a central inoculation point. Chemotaxis toward root cap cells and root tips is expressed as the chemotaxis ratio; the actual distance moved (in mm) toward the attractant, over the distance moved in the opposite direction, is shown in parentheses. Chemotaxis only toward sugars and amino acids that attracted A348 (threonine, glutamine, proline, histidine, asparagine, alanine, glucose, fructose, arabinose, sucrose, xylose, and mannose) was tested. The reaction was considered positive if the swarm moved farther in the direction of the attractant than in the opposite direction.

[b] C503 was attracted to sucrose, but not to any other sugars.

[c] C503 was attracted to glutamine, histidine, and asparagine.

[d] C1786 was attracted to all amino acids that attract A348.

[e] C294 was attracted to arabinose and sucrose.

[f] C294 was attracted to all of the amino acids that attract A348.

suspensions of *Chlorella* cells and that the bacteria are attracted to root tips but not to root cap cells of several different plant species.

We were able to isolate several Tn*5* mutants deficient in chemotaxis to exudates from root cap cells and excised root tips. The relatively high yield of mutants (11/1,500) and the diversity of mutant phenotypes suggests that multiple genes control the behavior. The independence of root exudate chemotaxis from the Ti plasmid suggests that chemotaxis is encoded chromosomally, although it does not rule out involvement of Ti plasmid genes in the process. None of the mutations appear to involve severe derangements of the cell surface; several of the motile mutants can respond chemotactically to some chemicals, and even the nonmotile mutants can bind as well as the parent to root cap cells. Nevertheless, all of the mutants that were deficient in chemotaxis to roots and root cap cells were deficient in chemotaxis to a variable range of sugars and amino acids, suggesting that a defect in recognizing individual chemicals may not eliminate the ability of the bacteria to respond to all components of root exudates. Although we do not yet know the nature of the attractive compounds or mixture of compounds in root exudates, it is interesting to note that although two of the chemotaxis mutants were attracted to all of the amino acids that attract strain A348, none of the chemotaxis mutants were attracted to all of the sugars that attract the parent. In *E. coli*, mutants that are deficient in chemotaxis to groups of related chemicals have been shown to have defects in gene products that transmit information from specific chemoreceptors to flagella (Adler 1975; Parkinson 1981).

The isolation of one mutant, C586, which is attracted to excised root tips but not root cap cells, supports the suggestion that attractants from sloughed cells are distinct from those from wounded tissue. The elucidation of the bases for the difference could yield insight into how pathogens differentiate between exudates from healthy tissue, which include sources of carbon and nitrogen for growth, and wounded tissue, a potential site of infection. In similar studies, slow-migrating and nonmotile mutants of *R. japonicum* and *R. meliloti* were found to be capable of inducing nodules on their respective hosts (Ames and Bergman 1981; Ames *et al.* 1980; Hunter and Fahring 1980). Interestingly, however, their effectiveness in competition studies is significantly reduced. It should be possible to use our mutants to determine if chemotaxis in *A. tumefaciens* plays an important role in pathogenesis of host plants and in rhizosphere colonization.

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