Chemotaxis of fluorescent *Pseudomonas* spp. to soybean seed exudates *in vitro* and in soil

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Five seed-colonizing fluorescent *Pseudomonas* strains (RW1 to RW5) exhibited chemotaxis toward soybean seed exudates in 1-μL capillaries held for 30 min in an 8.0 log colony-forming units/mL bacterial suspension over the temperature range of 9 to 41°C. Dialysis (6000 molecular weight cut-off) of exudate nullified its attractiveness to RW1; heating (121°C, 15 min) of exudate had no effect. Several amino acids present in exudate induced a chemotactic response by RW1, and asparagine, threonine, and valine at levels in exudate were as attractive as exudate. No chemotaxis by RW1 was observed toward sugars present in exudate. RW1 to RW5 actively migrated 1 cm toward soybean seeds in soil as demonstrated by a new method. A nonmotile mutant of RW3 did not migrate in soil and no significant migration by strains was observed when no seed or exudate was present. It is suggested that chemotaxis of *Pseudomonas* toward seed exudates may be the first step in establishment of bacterial seed and root colonization in soil.

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Cinq souches fluorescentes de *Pseudomonas* (RW1 à RW5), colonisatrices de grains de semence, ont présenté une chimiotaxie face à des exsudats de graines de soya dans des capillaires de 1 μL maintenus durant 30 min dans une suspension bactérienne de 8.0 log unités formant des colonies/mL selon une gamme de températures de 9–41°C. La dialyse (limite de poids moléculaire 6000) de l'exsudat a annulé son attraction pour le RW1; un chauffage de l'exsudat à 121°C, 15 min, n'a eu aucun effet. Plusieurs des acides aminés présents dans l'exsudat ont induit une réponse chimiotaxique chez le RW1; de plus, des niveaux égaux d'asparagine, de thréonine et de valine dans l'exsudat se sont avérés aussi attractifs que l'exsudat lui-même. Le RW1 n'a présenté aucune chimiotaxie pour les sucres présents dans l'exsudat. Les RW, de 1 à 5, ont migré activement de 1 cm vers des graines de soya dans du sol, tel que démontré par une nouvelle méthode. Un mutant non motile de RW3 n'a pas fait de migration dans le sol; de plus, aucune migration des souches n'a été observée en absence de graines ou d'exsudat. Il est donc ici suggéré que la chimiotaxie chez *Pseudomonas* face aux exsudats de graines peut être la première étape dans l'établissement d'une colonisation bactérienne des graines et des racines dans le sol.

[Traduit par le journal]

Motility and chemotaxis are common attributes of many bacteria and may be important for bacterial detection of available nutrients in natural environments. There is intense bacterial competition for nutrients in soil, and some strains exhibit chemotaxis toward nutrient-rich regions (Chet and Mitchell 1976). For example, *Pseudomonas* spp. exhibited chemotaxis in soil toward fungal exudates (Arora et al. 1983). *Rhizobium meliloti* moved through steam-sterilized soil in response to a general nutrient gradient created by utilization of substrates by the bacteria (Soby and Bergman 1983). *Rhizobium* spp. exhibited specific chemotaxis *in vitro* to a glycoprotein which was present in root exudates of bird's-foot trefoil (Strobel and Currier 1976; Currier and Strobel 1977). Such chemotaxis provided *R. meliloti* a competitive advantage over nonmotile strains (Ames and Bergman 1981) and increased nodulation by *R. japonicum* in greenhouse tests (Hunter and Fahring 1980).

Bacteria isolated from the rhizoplane of marine eelgrass exhibited chemotaxis *in vitro* to root exudates (Wood and Hayasaka 1981), and nitrogen-fixing *Azospirillum* strains exhibited chemotaxis to specific amino acids found in exudates of graminacious roots (Alvarez-Moralez and Lemos-Pastiana 1980). Attraction to root exudates may partially account for the high root-colonizing capacity of certain rhizosphere bacteria (Scher, Ziegle, and Kloepper 1984).

The first step in such bacterial root colonization is the rapid colonization of the germinating seed which profusely exudes a wide range of amino acids, carbohydrates, organic acids, etc., during germination (Hayman 1969; Stanghellini and Hancock 1970; Balasubramanian and Rangaswami 1978; Lynch 1978). Chemotaxis toward nutrient-rich seed exudates may represent a competitive advantage for certain bacteria in early establishment on the seed and radicle; however, no reports are available at this time on chemotaxis of bacteria specifically toward seed exudates. The objective of this investigation is to determine if *Pseudomonas* spp. native to soil exhibit chemotaxis to seed (soybean) exudates. As occurrence of chemotaxis *in vitro* does not establish the phenomenon *in situ*, a method is described to assess bacterial chemotaxis to seeds and seed exudates directly in soil. A preliminary report of the results was published (Scher, Kloepper, and Singleton 1984).

Materials and methods

Bacterial strains

Fluorescent *P. putida* (RW1 and RW3) and *P. fluorescens* (RW2, RW4, and RW5) were isolated from fallow soils or soybean rhizosphere soil (strain RW1) on *Pseudomonas* agar F (PAF) (Difco, Detroit, MI, U.S.A. 48232), and spontaneous rifampicin-resistant mutants were selected.

A Tn5-induced nonmotile mutant of RW3 was employed for comparative study. To obtain a nonmotile mutant, *P. putida* RW3 (Rif^r) was surface mated on PAF with *Escherichia coli* WA803 containing the transmissable plasmid pCU1::Tn5 (Ap^r, Sp^r, Sm^r, Km^r, tra^+) (Konarska-Kozlowska and Iyer 1981) for 1 h at 30°C. Transconjugants were retrieved on PAF containing 100 μg rifampicin and 60 μg kanamycin/mL and screened for negative motility and chemotactic attraction to soybean seed exudates in soft agar (method described below). $RW3^-$ was nonmotile when viewed microscopically.

Bacteria were stored in 50% glycerol at −80°C until 24 h prior to use, when they were inoculated into glycerol salts medium (Adler 1973) and incubated (shaking at 100 rpm) for 24 h at 30°C. Cells were rinsed twice and suspended in 10^{-2} *M* phosphate buffer, pH 7.0 (PB).

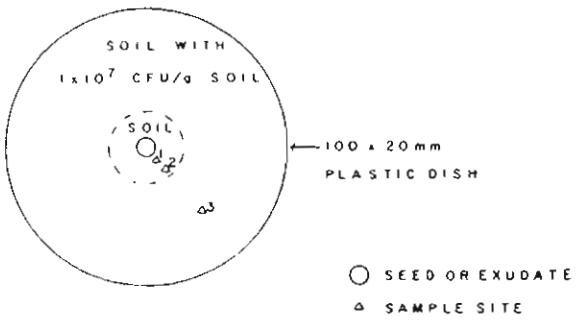

FIG. 1. Diagramatic representation of apparatus used to detect bacterial chemotaxis over 1 cm in soil. The inner ring (– – –) was removed after inner and outer soil chambers were wetted to allow movement of bacteria across the soil. Control plates contained no seed or exudate. Soil samples were taken from sites 1, 2, and 3. The apparatus differed slightly to detect chemotaxis over 2 cm: sites 1, 2, and 3 were in the inner chamber and site 4 was in the outer chamber (see Fig. 4).

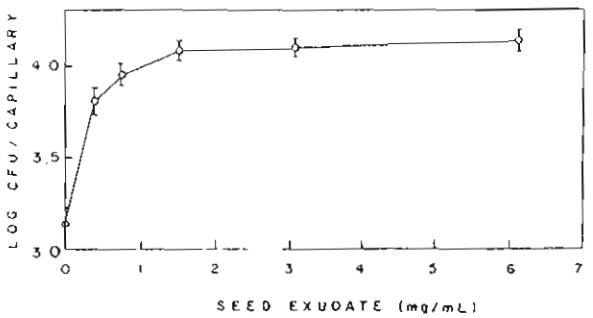

FIG. 2. Numbers of *Pseudomonas putida* (RW1) in 1-μL capillary tubes containing varying concentrations of soybean seed exudate rehydrated (after freeze-drying) in 0.01 *M* phosphate buffer (pH 7.0). Capillaries were held in an 8.0 log cfu/mL RW1 suspension for 30 min at 23°C.

Seed exudate

Soybean (cv. Maple Presto) seeds were surface sterilized by soaking in 70% ethanol for 20 min then in 20% bleach for 35 min, and rinsed thoroughly with sterile H_2O. Five seeds were added to sterile 50-mL Erlenmeyer flasks containing 5 mL of sterile H_2O. Flasks were incubated (shaking, 60 rpm) at 25°C for 5 days, after which exudate was collected and sterilized by filtration (0.20 μm). Sterile exudate was stored at 4°C or freeze-dried and stored at −20°C. Freeze-dried exudate was rehydrated in PB. Unless otherwise noted, exudate refers to nonfreeze-dried exudate. Exudate was autoclaved 30 min (120°C, 15 psi (1 psi = 6.895 kPa)) or dialyzed (6000 molecular weight cut-off tubing, in H_2O) for diagnostic purposes.

Compounds present in exudate were assessed by two methods. First, free amino acids were detected using a Beckman (model 121) amino acid analyzer; a 56 cm AA15 ion-exchange resin in 1 *M* sodium citrate buffer was employed. Secondly, sugars were detected by spotting 5 μL concentrated freeze-dried exudate (15 mg/mL) onto silica gel thin-layer chromatography (TLC) plates (Baker Chemical Co., Phillipsburg, NJ, U.S.A. 08865), after which plates were held in 90% acetone for 1.5 h, dried, sprayed with anisaldehyde − sulfuric acid (Stahl and Kaltenback 1961), and heated 10 min at 100°C. Comparisons of spot colours and R_f values were made to 10^{-3} *M* sugar standards.

Chemotaxis in vitro

In vitro chemotaxis to seed exudate was assessed by two methods. First, bacteria from a 24-h PAF culture were spotted onto soft agar (0.2%) containing seed exudate (5%) and the diameters of resulting swarms were determined after 24 h incubation at 23 ± 1°C. Secondly, a modification of Adler's capillary method (Adler 1973) was employed. Capillary tubes (1 μL) (Drummond Scientific Co., Broomall, PA, U.S.A. 19013) were heat-sealed on one end and filled with test attractant or PB (control). Individual capillaries were placed in 12 × 75 mm polystyrene test tubes containing 0.5 mL of a 1 × 10^4 colony-forming units (cfu) per millilitre of baeterial suspension, and incubated 30 min at 23 ± 1°C in the standard assay. Capillaries were rinsed with 95% ethanol, then H_2O, and broken at the sealed ends. Contents were ejected into 2-mL 0.1 *M* $MgSO_4$ blanks, mixed, and plated onto PAF containing 100 μg rifampicin/mL (PAF-R) with a spiral plater (Spiral Systems, Bethesda, MD, U.S.A. 20814). Eight replicates per treatment were used; enumerations were made after 48 h incubation at 30°C and cfu per capillary were calculated. These methods were used to test the attraction of RW1 to exudate or L isomers of amino acids dissolved in PB.

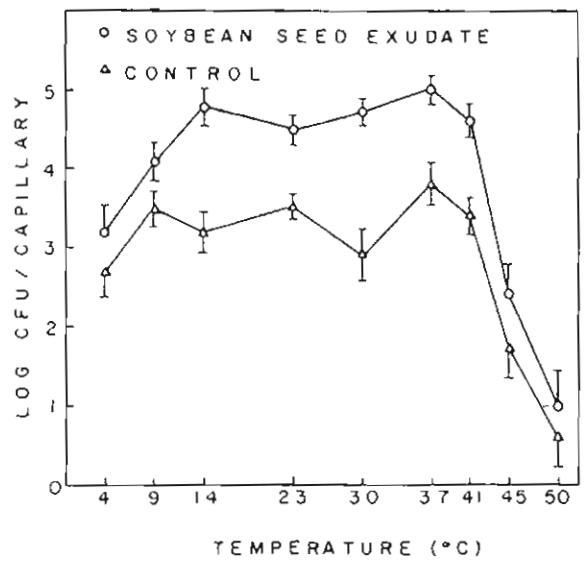

FIG. 3. Numbers of RW1 cfu in 1-μL capillary tubes containing soybean seed exudate or 0.01 *M* phosphate buffer, pH 7.0 (control), after capillaries were held in an 8.0 log cfu/mL RW1 suspension for 30 min at various temperatures.

TABLE 1. Chemotaxis of *Pseudomonas putida* (RW1) to treated exudate

Compound	cfu/capillary (log)
Control[a]	3.9
Exudate	4.7[d]
Autoclaved exudate[b]	4.8[d]
Dialyzed exudate[c]	3.9

[a]Potassium phosphate buffer (10^{-3} *M*), pH 7.0.
[b]Autoclaved at 121°C, 20 min.
[c]Dialyzed using 6000 molecular weight cut-off tubing, freeze-dried, and rehydrated to 1 mg dry matter/mL.
[d]Significantly different from control ($P = 0.01$).

Chemotaxis in soil

Chemotaxis of bacteria toward seed and seed exudate in a sieved (4 mm), raw loam soil (pH 6.8) was assessed using the apparatus in Fig. 1. Soil amended with 7.0 log cfu rifampicin-resistant bacteria/g soil was held in the outer ring of a 25 × 100 mm petri dish holding a 1 or 2 cm radius (r) × 20 mm deep interior ring. Soil in the centre ring contained no detectable rifampicin-resistant bacteria and was not amended with them. Either a preimbibed soybean seed or 100 μL of concentrated (30 mg/mL) freeze-dried exudate was placed in the centre ring (at 1 cm depth), and covered with soil. Total soil was approximately 70 g/plate and was adjusted to 30% moisture by addition of water to both compartments of soil. The inner ring barrier was removed and the plates were tamped lightly to seal the fissure.

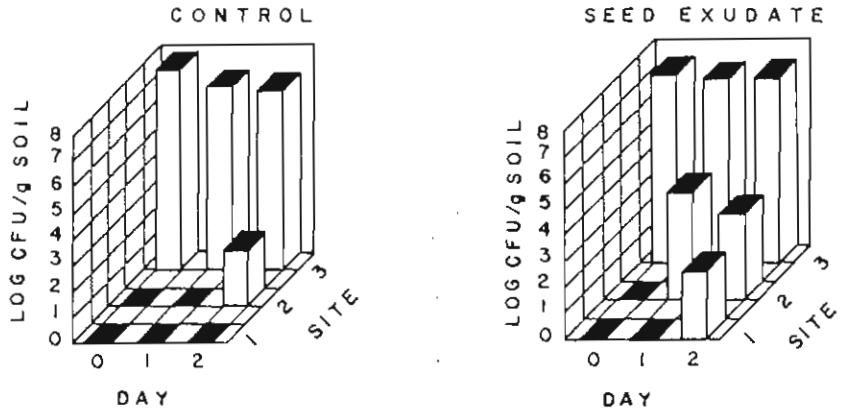

FIG. 4. Numbers of RW1 cfu in samples taken from the soil chemotaxis apparatus (1 cm *r* inner ring), with and without seed exudate, over time. (See Fig. 1 for site description.)

TABLE 2. Chemotaxis of *Pseudomonas putida* (RW1) to amino acids at levels in soybean exudate

Compound	Concn. (log *M*)	cfu/capillary (log)
Control	−2.0[a]	3.1
Exudate	—	4.6
Gln	−4.2	3.1
Glu	−4.2	3.2
Ser	−4.5	3.3
Ala	−4.4	3.8[b]
Asn	−4.6	4.0[c]
Asp	−4.6	3.2
Lys	−4.9	2.5[b]
Val	−5.0	3.9[c]
Thr	−5.0	4.0[c]
Leu	−5.0	3.7[b]
Pro	−5.0	3.8[b]
Ile	−5.1	3.5
Cys	−5.1	3.7[b]
Arg	−5.1	2.1[b]
Phe	−5.3	3.8[b]
Tyr	−5.3	3.5
Gly	−5.4	3.8[b]
His	−6.0	3.4

[a]Potassium phosphate buffer, pH 7.0.
[b]Significantly different from control ($P = 0.01$).
[c]Significantly different from control but not from exudate ($P = 0.01$).

TABLE 3. Chemotaxis of *Pseudomonas putida* (RW1) to amino acid mixtures

Compound	cfu/capillary (log)
Control[a]	3.9
Exudate	4.9[c]
AA + Asp + Gln[b]	4.9[c]
AA + Asn + Gln	4.9[c]
AA + Asn + Glu	4.9[c]
AA + Asp + Glu	4.9[c]

[a]Potassium phosphate buffer (10^{-2} *M*), pH 7.0.
[b]AA = mixture of amino acids in Table 2 at levels in exudate, except Asn, Asp, Gln, or Glu, which were added in the combination shown.
[c]Significantly different from control ($P = 0.01$).

Soil samples were taken from the centre (site 1), just inside of the outer ring (site 2), or in the outer ring (site 3) by vertically inserting a narrow spatula into the tared plate and withdrawing 0.1 g soil into a 10-mL 0.1 *M* $MgSO_4$ blank. Samples were taken from four replicate plates at

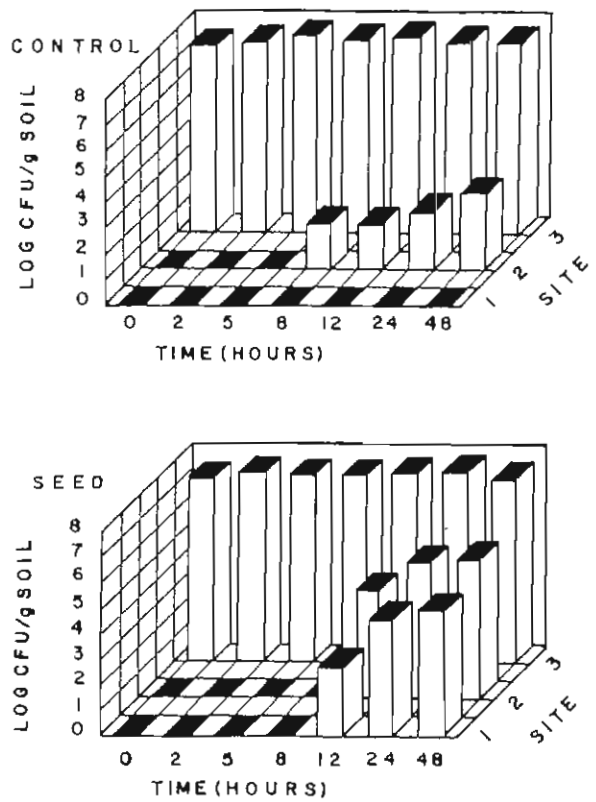

FIG. 5. Numbers of RW1 cfu in samples taken from the soil chemotaxis apparatus (1 cm *r* inner ring), with and without soybean seeds over time. (See Fig. 1 for site description.)

time zero and after 24 and 48 h for 1 cm *r* rings, or after 24, 48, and 72 h for 2 cm *r* rings. Each bacterial treatment included a control in which neither exudate nor a seed was present in the centre.

Results

Chemotaxis in vitro

Pseudomonas putida (RW1) exhibited chemotaxis toward soybean exudate in soft (0.2%) agar. The average swarm diameter after 24 h was 50 mm; no swarm was evident when exudate was omitted from the medium. RW1 also exhibited chemotaxis toward rehydrated freeze-dried exudate in the capillary assay (Fig. 2). A significant response was observed with 0.5 mg dry matter/mL, and 1.5 mg/mL gave the maximum response. Standard exudate, before freeze-drying, contained 3 mg/mL.

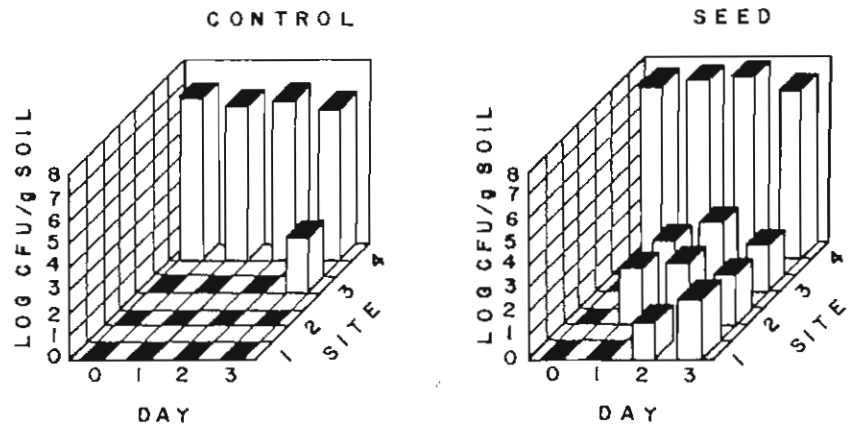

FIG. 6. Numbers of RW1 cfu in samples taken from the soil chemotaxis apparatus (2 cm *r* inner ring), with and without soybean seeds, over time. (See Fig. 1 for site description.)

The effect of temperature on chemotactic attraction by RW1 to exudate was determined with the capillary assay. Significant attraction, compared with phosphate buffer controls, occurred from 9 to 41°C (Fig. 3). This was the same range over which general motility (as evidenced by control capillaries) was retained. Subsequent capillary assays were performed at 23°C.

Autoclaving exudate did not affect its attractiveness; however, dialyzing exudate nullified its attractiveness (Table 1). This indicated that the attractive compound(s) was neither proteinaceous nor greater than 6000 daltons.

From TLC analysis, exudate was found to contain the sugars sucrose, fructose, galactose, rhamnose, and glucose. Precise concentrations were not determined, but all sugars were greater than 10^{-4} *M* in standard exudate. RW1 did not exhibit significant chemotaxis to any of the five sugars at 10^{-3} or 10^{-2} *M* in the capillary assay.

Eighteen amino acids, all at concentrations less than 6×10^{-5} *M*, were found in the soybean seed exudate (Table 2). RW1 exhibited significant positive chemotaxis to asparagine, valine, threonine, leucine, proline, cysteine, phenylalanine, and glycine at levels present in exudate (Table 2). Arginine and lysine were repellent at levels in exudate. Attraction to asparagine, valine, and threonine was not significantly different than to exudate. At higher concentrations (10^{-3} *M*), RW1 was attracted to all amino acids found in exudate except lysine, aspartic acid, gutamic acid, and arginine (data not shown).

RW1 exhibited chemotaxis to mixtures of amino acids at their levels in exudate (Table 3), and varying the combinations of aspartic acid, asparagine, glutamic acid, and glutamine in the mixture had no effect on attractiveness. These amino acid mixtures produced a chemotactic response approximately equal to that of the exudate.

Soil chemotaxis

RW1 remained at approximately 7.0 log colony-forming units (cfu)/g soil in the outer ring (the inoculation site) of the soil chemotaxis apparatus (Fig. 1) for the duration of all experiments (Figs. 4–6). RW1 was occasionally detected in site 2, but not site 1, of control plates.

When seed exudate was present in the inner soil ring, RW1 migrated into site 2 after 1 day and was present (2.5 log cfu/g soil) in site 1 after 2 days (Fig. 4). A somewhat stronger response was noted toward soybean seeds placed in the centre; RW1 (4.5 log cfu/g soil) was found after 24 h in the inner ring of 1 cm *r* rings containing seeds (Fig. 5). Earliest detected migration over the 1 cm of soil was after 12 h.

TABLE 4. Comparative bacterial chemotaxis in soil, capillary, and soft agar assays

Strain	cfu/g soil (log)[a]		cfu/capillary (log)		Soft agar, diameter (mm)[b]
	Control	Seed	Control	Exudate	
RW1	N[c]	5.2[d]	3.9	4.8[d]	50[d]
RW2	N	5.5[d]	2.9	4.1[d]	55[d]
RW3	N	5.6[d]	2.5	3.3[d]	30[d]
RW3$^-$	N	N	1.5	0.8	4
RW4	N	5.6[d]	3.0	3.9[d]	25[d]
RW5	N	5.1[d]	3.5	4.5[d]	80[d]

[a] Represents the number of bacteria reaching the inner ring (site 1, 1.0 cm) of soil chemotaxis plates after 48 h.
[b] Agar (0.2%), 5% exudate; diameters on control plates (no exudate) were 0–4 mm.
[c] N, not detected.
[d] Significantly different from respective control ($P = 0.01$) within the same assay.

An experiment was performed utilizing larger (2 cm) *r* rings to determine whether the bacteria were capable of detecting seeds 2 cm away. The response was lower than with 1-cm rings, but RW1 did migrate 2 cm toward seeds and was present (1.4 log cfu/g soil) in the inner ring after 2 days.

Comparative chemotaxis

Chemotaxis by six fluorescent *Pseudomonas* spp. (one nonmotile) was compared in the three assays. All showed strong attraction to soybean seeds in soil except the nonmotile RW3$^-$ (Table 4). Population densities were either greater than 5.0 log cfu/g soil in site 1 (1 cm *r* plates) after 2 days, or were not detected.

The same differential response was seen between strains with the capillary assay. RW3$^-$ showed little migration into capillaries and no significant response ($P = 0.01$) to exudate. All strains except RW3$^-$ showed significant migration in soft agar containing exudate ($P = 0.01$), as compared with that with no exudate. RW5 showed the highest response, with a swarm diameter of 80 mm after 24 h.

Discussion

Fluorescent pseudomonads used in this study exhibited chemotaxis *in vitro* to both soybean seed exudate and specific amino acids found in the exudate. In addition, a previously unreported technique was developed and used to confirm that these pseudomonads exhibited chemotaxis toward exudates in a raw field soil.

Chemotactic attraction of *P. putida* strain RW1 to asparagine, valine, and threonine at levels in seed exudate (Table 2) or to a mixture of amino acids (Table 3) was not significantly different than to exudate, indicating that amino acids are responsible for seed exudate attractiveness.

Strain RW1 was not attracted *in vitro* to sugars present in soybean seed exudate (sucrose, glucose, fructose, galactose, or rhamnose), confirming earlier work with a *P. fluorescens* strain (Seymour and Doetsch 1973). However, this *P. fluorescens* differed from *P. putida* RW1 in attraction to specific amino acids. RW1 was attraced to glycine, histidine, leucine, proline, and tryptophan (at 10^{-2} *M*), whereas the reported *P. fluorescens* was not. Thus, response to amino acids and, ultimately, exudates may vary among closely related bacteria.

Pseudomonas putida strain RW1 migrated 1 cm in 12 h (Fig. 5) and 2 cm in 48 h (Fig. 6) toward individual imbibing soybean seeds in raw (nonsterilized) soil. The inability to detect RW1 in site 1 of control plates or a nonmotile mutant of RW3 in the vicinity of imbibing seeds confirmed that the soil chemotaxis procedure detected bacteria which entered site 1 as a result of chemotaxis rather than passive movement in the soil solution.

Bacterial population densities (Figs. 4–6) detected at various sample times at sites 1 and 2 of the soil chemotaxis assay (Fig. 1) do not necessarily equate with the population of incoming, chemotaxing bacteria, but are likely to include bacteria which multiplied inside site 1 after the initial chemotaxis. While chemotaxis in soil toward seed exudates may confer a competitive advantage for seed colonization by bacteria, rapid multiplication may also provide a competitive advantage for bacteria, once within the spermosphere. Thus, regardless of the relative role of actual chemotaxis or "postchemotaxis" multiplication, the soil chemotaxis assay indicated the competitive ability of the tested bacteria in reaching and colonizing seeds.

Bacterial attraction to exudates *in vitro* occurred over a broad temperature range (Fig. 3), indicating that temperature would not be a limiting factor for chemotaxis in agricultural systems. Soil moisture was at saturation in the soil chemotaxis assay and it was not determined if bacteria can migrate through soil under drier conditions. One might suggest that moisture sufficient for seed imbibition would be sufficient for movement of bacteria in microcapillaries through soil or that periodic rain or irrigation would result in an environment conducive to chemotaxis-mediated migration toward seeds.

Seed colonization can result in subsequent root colonization of developing plants as evidenced by the root colonization capacity of some pseudomonads inoculated onto maize seeds (Scher, Ziegle, and Kloepper 1984). It is also likely that chemotaxis to root exudates may be involved in migration of bacteria from seeds to roots, and our preliminary results indicate that RW1 also exhibits chemotaxis to soybean root exudates, which are chemically distinct from seed exudates. We propose that chemotaxis to seed and root exudates is one of several traits which constitutes a successful seed- and root-colonizing bacterium.

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