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Chemotaxis of *Azospirillum* Species to Aromatic Compounds

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Chemotaxis of *Azospirillum lipoferum* Sp 59b and *Azospirillum brasilense* Sp 7 and Sp CD to malate and to the aromatic substrates benzoate, protocatechuate, 4-hydroxybenzoate, and catechol was assayed by the capillary method and direct cell counts. *A. lipoferum* required induction by growth on 4-hydroxybenzoate for positive chemotaxis to this compound. Chemotaxis of *Azospirillum* spp. to all other substrates did not require induction. Maximum chemotactic responses for most aromatic compounds occurred at concentrations of 1 to 10 mM for *A. lipoferum* and 100 μM to 1 mM for *A. brasilense*. Threshold levels of these chemoattractants ranged from nanomolar to micromolar, with *A. brasilense* Sp CD showing the lowest threshold levels for the substrates tested. Benzoate was the strongest chemoattractant tested, with threshold concentrations in the nanomolar to picomolar range for all strains. *Azospirillum* spp. clearly have more sensitive chemosensory mechanisms for certain aromatic substrates than previously reported in some other soil bacteria. This sensitivity allows *Azospirillum* spp. to detect and respond to aromatic substrates at concentrations relevant to the soil and rhizosphere environments. The ability to detect such low concentrations of aromatic compounds in soils may confer advantages in survival and colonization of the rhizosphere by *Azospirillum* species.

Azospirillum species are free-living nitrogen-fixing bacteria commonly found in soils and in association with roots of plants, including such important agricultural crops as rice, maize, wheat, and legumes (4, 6, 13, 14, 32). Rhizosphere colonization by *Azospirillum* species has been shown to stimulate growth of a variety of plant species, although the basis for this stimulation is not clear at present (6). The success of the *Azospirillum*-plant interaction depends on the survival and persistence of these bacteria in soil and the effective colonization of the rhizosphere. Chemotaxis is one of several properties which may contribute to survival, rhizosphere colonization, and the initiation of mutualistic interactions by *Azospirillum* species (6, 28).

Chemotaxis allows bacteria to respond to chemical gradients, seeking higher levels of potential nutrients and lower levels of inhibitors (1). In addition to their utility in foraging for growth substrates, chemotactic responses of soil bacteria to aromatic compounds have been implicated in the establishment of some well-studied plant-microbe interactions (7, 8, 11, 20, 23). Luteolin induces nodulation genes in *Rhizobium meliloti* and is also a chemoattractant to this organism (11). Chemotaxis to methoxyphenols such as acetosyringone appears to play a role in the initiation of the parasitic interaction between *Agrobacterium* spp. and plants (23). However, the contribution chemotaxis makes to survival of soil bacteria and initiation of plant-microorganism interactions is still not well understood. Of particular interest is the ability of soil bacteria to respond to in situ substrate concentrations.

Chemoattraction of *Azospirillum* species to sugars and organic acids has been reported (5, 28), but responses to aromatic compounds have not been examined. A variety of aromatic compounds found in soils, sediments, and the rhizosphere (33, 34, 35) can be utilized by *Azospirillum* species as carbon and energy sources (9), but these compounds occur at very low (10^{-8} M) concentrations in many cases (35). Chemoattraction of other common soil bacteria, including *Bradyrhizobium* spp., some *Pseudomonas* species, and *Rhizobium* spp. to aromatic compounds has been reported previously (18, 19, 26), but threshold concentrations are often around 50 μM, implying low sensitivity of these organisms to gradients of aromatic substrates at environmentally relevant concentrations. In this study, we have evaluated the chemotactic responses of three *Azospirillum* strains to a variety of aromatic compounds. Some of these compounds are strong chemoattractants of *Azospirillum* species when supplied at levels below the detection thresholds reported for other soil bacteria and well within the concentration ranges reported for soils.

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MATERIALS AND METHODS

Bacterial strains and culture conditions. *Azospirillum lipoferum* Sp 59b and *Azospirillum brasilense* Sp 7 and Sp CD were obtained from Peter van Berkum, U.S. Department of Agriculture. Cultures were incubated with gentle agitation at 28°C in a medium derived from NBS minimal medium (21). This medium contained the following (per liter of distilled water): $MgSO_4 \cdot 7H_2O$, 0.2 g; $(NH_4)_2SO_4$, 0.4 g; NaCl, 0.1 g; $CaCl_2$, 0.02 g; Bacto Yeast Extract, 0.1 g; $NaMoO_4 \cdot 2H_2O$, 0.04 mg; $MnSO_4 \cdot H_2O$, 0.47 mg; H_3BO_3, 0.56 mg; $CuSO_4 \cdot 5H_2O$, 0.016 mg; $ZnSO_4 \cdot 7H_2O$, 0.048 mg; biotin, 0.01 mg; and pyridoxine, 0.02 mg. The medium had a final pH of 6.8. The medium was buffered with 3 mM morpholinepropanesulfonic acid (MOPS) and supplemented with 0.5% (37.3 mM) DL-malate, 2 mM 4-hydroxybenzoate (4-HB), or 2 mM protocatechuate (3,4-dihydroxybenzoic acid; PCA). Exponentially growing cultures were harvested by centrifugation, and the hanging drop method was used to confirm cell motility. Cell suspensions were prepared with 60 mM potassium phosphate buffer (pH 6.8; KPB) (28) without EDTA at a final density of 10^7 cells per ml. More than 90% of the cells remained motile after these procedures.

Chemotaxis assay. A modification of the capillary method (1, 2) and a chemotaxis chamber with four separate compartments (24) were used for chemotaxis assays. Each compart-

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TABLE 1. Chemotactic responses of *Azospirillum* spp. to BA[a]

Concn	Mean CI[b]		
	A. lipoferum Sp 59b	*A. brasilense* Sp 7	*A. brasilense* Sp CD
10 mM	2.65 (0.50)	2.07 (0.54)	6.04 (2.18)
1 mM	2.83 (0.49)	3.22 (0.70)	7.46 (2.00)
100 μM	3.74 (0.86)	3.51 (0.76)	6.80 (1.58)
10 μM	4.52 (0.62)	3.34 (0.92)	7.18 (1.94)
1 μM	3.06 (0.55)	2.91 (0.75)	9.91 (1.35)
100 nM	2.34 (0.50)	2.55 (0.61)	3.72 (0.34)
10 nM	1.91 (0.17)	2.43* (0.31)	3.42 (0.04)
1 nM	1.77 (0.20)	1.30 (0.26)	1.68 (0.16)
100 pM	1.86* (0.19)	1.03 (0.21)	1.40* (0.06)
10 pM	1.25 (0.23)	ND[c]	1.04 (0.06)

[a] Cells were grown on NBS minimal medium supplemented with 0.5% DL-malate and prepared for experiments as described in Materials and Methods.

[b] The CI is calculated as the ratio of cell density in experimental capillaries to that in control capillaries (see Materials and Methods). Data are reported as mean CI with one standard deviation shown in parentheses. Peak response values are boxed and threshold levels are indicated by asterisks ($P < 0.05$ for all experiments by ANOVA).

[c] ND, not determined.

ment consisted of two wells interconnected by a channel. Three-microliter microcapillary tubes (Drummond Scientific Co., Broomal, Pa.) containing the test solutions were inserted in the channels with their ends touching the motile cell suspensions contained in the wells of the chambers. Bacteria migrated to the inside of the capillary if attracted to the test solution but not if repelled by it. Oxygen microelectrode measurements of oxygen levels within and outside the capillaries showed no significant changes in oxygen levels during the course of the experimental incubation, thus ruling out aerotaxis as a source of experimental error. The aromatic compounds used, i.e., benzoate (BA), catechol (1,2-dihydroxybenzene; CAT), 4-HB, and PCA were obtained from Sigma (St. Louis, Mo.). Malate, a known chemoattractant of *Azospirillum* species (28), served as a positive control. The test chemical solutions were serially diluted in KPB, yielding a concentration range of 100 mM to 1 μM or lower as required to determine threshold concentrations. Eight replicate capillaries of each compound concentration and of KPB diluent were used, one in each of the four channels in each of the two chambers used per treatment. KPB served as a control for potential diluent effects on bacterial responses. After 1 h of incubation at 28°C, the contents of the capillaries were transferred to the wells of toxoplasmosis slides (Cel-Line Associates, Newfield, N.J.), heat fixed, and stained with 0.1% acridine orange for 1 min.

Cell numbers were determined by epifluorescence direct counting with an Olympus model BH2 microscope equipped for phase-contrast and epifluorescence applications. A minimum of 10 fields, containing at least 20 cells each, were counted for each well. Thus, for each treatment, 80 fields totaling a minimum of 1,600 cells were counted. The direct count method is a rapid and more accurate alternative to plate counts for bacterial enumeration in this type of experiment (22). The mean and one standard deviation of the cell density in eight replicates were then calculated. To facilitate comparison between experiments having differences in control cell density values, the ratio of bacterial density in the experimental assay microcapillaries to the density in the buffer controls (chemotactic index [CI]) was also calculated. A CI value of 1 indicates no response, a value greater than 1 indicates a positive chemotactic response, and a value less than 1 indicates a negative response. The mean and one standard deviation of the CI for eight replicate experiments were calculated.

Statistical analysis of data. Analysis of variance (ANOVA, Statgraphics; Statistical Graphics Corp., Rockville, Md.) was used to determine statistically significant differences between cell densities found in the experimental capillaries and those found in the buffer control capillaries. ANOVA was always performed with the raw cell density values and not the normalized CI values. Significant differences between responses were determined by the Student-Newman Keuls test and used to estimate threshold levels. Peak and threshold responses were used as described previously (2). Each concentration of each compound tested was examined in at least two separate experiments.

RESULTS

All three *Azospirillum* strains used in these experiments responded significantly to all of the aromatic substrates and to malate. Different peak responses (concentrations at which the maximum chemotactic response occurred) and threshold concentrations (the concentration below which no significant response is seen) were observed among the different strains tested. The magnitudes of the responses were also significantly different. The CI, or ratio of experimental cell density to the control cell density, was used to compare results from different experiments. Typical bacterial densities observed in experimental capillaries were 4.10×10^5 ($n = 8$, standard deviation = 4.44×10^4) cells per ml versus control densities

TABLE 2. Chemotactic response of *A. lipoferum* Sp 59b to aromatic compounds and malate

Concn	Mean CI[a]				
	CAT	PCA	4-HB	4-HB grown[b]	Malate
100 mM	ND[c]	ND	1.14 (0.39)	1.09 (0.24)	2.38 (0.07)
10 mM	2.74 (0.22)	2.18 (1.25)	1.10 (0.65)	2.05 (0.25)	3.23 (0.46)
1 mM	1.81 (0.17)	3.36 (0.74)	0.56 (0.34)	1.31 (0.03)	1.83 (0.38)
100 μM	1.62 (0.25)	2.91 (0.61)	0.29 (0.30)	1.59 (0.03)	1.36 (0.10)
10 μM	1.42* (0.25)	2.29* (0.80)	0.17 (0.07)	1.18* (0.09)	1.26* (0.10)
1 μM	1.07 (0.11)	1.00 (0.09)	0.18 (0.11)	1.17 (0.11)	0.95 (0.18)

[a] The CI is calculated as the ratio of cell density in experimental capillaries to that in control capillaries (see Materials and Methods). Results are reported as mean CI with one standard deviation shown in parentheses. Peak response values are boxed, and threshold levels are indicated by asterisks ($P < 0.05$ for all experiments by ANOVA).

[b] All treatments except this one used cells grown on malate. This treatment used cells grown on 4-HB.

[c] ND, not determined.

TABLE 3. Chemotactic response of *A. brasilense* Sp 7 to aromatic compounds and malate[a]

Concn	Mean CI[b]			
	CAT	PCA	4-HB	Malate
100 mM	ND[c]	ND	1.17 (0.16)	1.20 (0.16)
10 mM	1.90 (0.12)	1.12 (0.09)	1.38 (0.21)	1.13 (0.16)
1 mM	2.21 (0.24)	1.33 (0.12)	1.58 (0.31)	1.78 (0.26)
100 μM	1.72 (0.18)	1.66 (0.08)	1.41 (0.29)	2.38 (0.22)
10 μM	1.48 (0.18)	1.36 (0.15)	1.22 (0.21)	1.75 (0.09)
1 μM	1.29* (0.09)	1.31* (0.22)	1.13* (0.17)	1.62* (0.19)
100 nM	1.10 (0.11)	1.15 (0.07)	ND	1.14 (0.10)

[a] All treatments used cells grown on malate.
[b] The CI is calculated as the ratio of cell density in experimental capillaries to that in control capillaries (see Materials and Methods). Results are reported as mean CI with one standard deviation shown in parentheses. Peak response values are boxed, and threshold levels are indicated by asterisks ($P < 0.05$ for all experiments by ANOVA).
[c] ND, not determined.

of 1.86×10^5 ($n = 8$, standard deviation = 1.24×10^4) cells per ml. This density is equivalent to a CI of 2.2.

BA, followed by PCA, was the preferred aromatic chemoattractant for all three strains (Table 1), with CAT and 4-HB eliciting somewhat lower responses (Tables 2 to 4). Responses to some of the aromatic substrates were higher than those obtained with malate (Tables 1 and 2). *A. brasilense* Sp CD exhibited the strongest response to BA (maximum CI = 9.91), followed by *A. lipoferum* Sp 59b (maximum CI = 4.52) and *A. brasilense* Sp 7 (maximum CI = 3.51; Table 1 and Fig. 1). Interestingly, malate-grown *A. lipoferum* cells were repelled by 4-HB (CI < 1), but when grown on 2 mM 4-HB, this organism showed a significant positive response to this compound (CI > 1; Table 2). All three strains grown on malate were positively chemotactic to aromatic compounds other than 4-HB. The threshold concentrations of aromatic substrates, except BA, for the *A. lipoferum* chemotactic response were around 10 μM (Table 2). Threshold concentrations for BA were substantially lower, around 100 pM to 10 nM (Fig. 1B and Table 1). Threshold levels for chemoattraction of both strains of *A. brasilense* by aromatic substrates occurred at lower levels (1 μM; Tables 3 and 4), as low as 100 pM for BA (Table 1).

Differences between the *Azospirillum* species were also seen when the cells were grown on 2 mM PCA as the sole carbon source. PCA-grown *A. lipoferum* was not chemotactic to PCA or CAT (CI < 1; Table 5). However, not only did PCA-grown *A. brasilense* Sp 7 remain positively chemotactic to these substrates, but in addition, the response increased in magnitude (Table 6). The maximum CI obtained from malate-grown *A. brasilense* was about 2, while the response from PCA-grown cells exceeded 5.

TABLE 4. Chemotactic response of *A. brasilense* Sp CD to aromatic compounds and malate[a]

Concn	Mean CI[b]			
	CAT	PCA	4-HB	Malate
100 mM	ND[c]	ND	1.78 (0.18)	3.61 (0.43)
10 mM	1.71 (0.20)	1.65 (0.19)	2.07 (0.19)	4.39 (0.51)
1 mM	1.84 (0.30)	3.71 (0.44)	2.04 (0.23)	3.83 (0.42)
100 μM	2.55 (0.27)	2.56 (0.35)	2.56 (0.11)	6.08 (0.41)
10 μM	1.75 (0.27)	1.83 (0.31)	1.79 (0.13)	6.85 (0.42)
1 μM	1.40* (0.22)	1.39* (0.18)	1.28* (0.13)	4.82 (0.47)
100 nM	0.80 (0.12)	1.33 (0.17)	ND	2.13* (0.33)

[a] All treatments used cells grown on malate.
[b] The CI is calculated as the ratio of cell density in experimental capillaries to that in control capillaries (see Materials and Methods). Results are reported as mean CI with one standard deviation shown in parentheses. Peak response values are boxed, and threshold levels are indicated by asterisks ($P < 0.05$ for all experiments by ANOVA).
[c] ND, not determined.

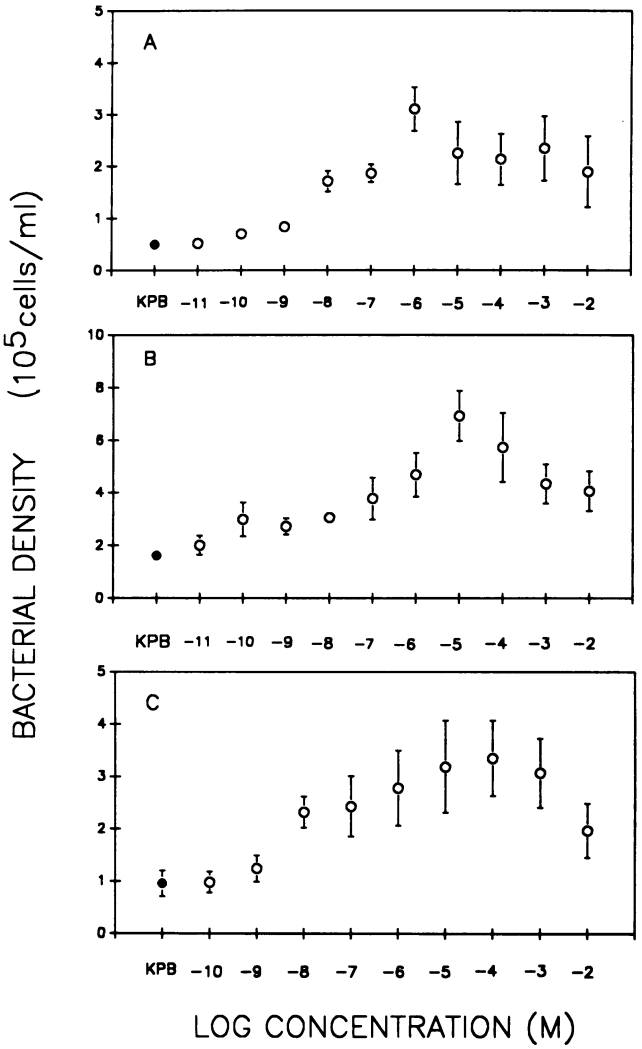

FIG. 1. Chemotactic responses of *A. lipoferum* Sp 59b (A), *A. brasilense* Sp 7 (B), and *A. brasilense* Sp CD (C) exposed to different concentrations of BA. Cells were grown on NBS minimal medium supplemented with 0.5% DL-malate and prepared for experiments as described in Materials and Methods. Error bars represent 1 standard deviation from the mean. Cell densities in KPB with no chemoattractants are given for comparison (filled circles). Symbols with no error bars represent data with standard deviations smaller than the symbol used.

DISCUSSION

Chemotaxis provides a means for bacteria to respond to environmental gradients of potential nutrients and toxins, resulting in directed motility toward or away from these substances (1–3). Aromatic compounds found in soils and in the rhizospheres of plants support growth of a wide variety

TABLE 5. Comparison of the chemotactic responses to PCA and CAT by malate- and PCA-grown *A. lipoferum* Sp 59b

Concn	Mean CI[a]			
	Malate-grown cells		PCA-grown cells	
	PCA	CAT	PCA	CAT
10 mM	2.18 (1.25)	2.74 (0.22)	0.82 (0.03)	0.81 (0.07)
1 mM	3.36 (0.74)	1.81 (0.17)	0.99 (0.01)	0.90 (0.09)
100 μM	2.91 (0.61)	1.62 (0.25)	0.84 (0.02)	0.69 (0.05)
10 μM	2.29* (0.80)	1.42* (0.25)	0.97 (0.06)	0.67 (0.06)
1 μM	1.00 (0.09)	1.07 (0.11)	0.87 (0.02)	0.96 (0.07)

[a] The CI is calculated as the ratio of cell density in experimental capillaries to that in control capillaries (see Materials and Methods). Results are reported as mean CI with one standard deviation shown in parentheses. Peak response values are boxed, and threshold levels are indicated by asterisks ($P < 0.05$ only for malate-grown cells by ANOVA). No significant differences were obtained in PCA-grown cells ($P > 0.05$).

of soil bacteria in pure culture (31) and are chemoattractants for some of these species (18, 26). The utilization of several aromatic compounds as carbon and energy sources by *Azospirillum* species was recently demonstrated (9), prompting us to examine *Azospirillum* chemotactic responses to these compounds. Several aromatic compounds occur naturally in soils at concentrations of around 10^{-8} M (35), so we were particularly interested to see if substrates at these low concentrations could elicit a chemotactic response.

As was shown for chemotaxis to certain sugars by *Escherichia coli* (3), chemoattraction to most aromatic compounds by *Azospirillum* species was dependent on the capacity of these organisms to catabolize these compounds. The chemoattractants BA, CAT, PCA, and 4-HB support growth and nitrogen fixation in *Azospirillum* species (9). Some of these responses were relatively low in magnitude but similar to those reported for *Azospirillum* species to organic acids, including succinate (28), and to that of *R. meliloti* to the phenolic derivative luteolin (11), among others (8). Although BA alters intracellular pH in enteric bacteria (29) and is a repellent to these organisms, it yielded the highest positive responses and the lowest threshold concentrations (10^{-8} to 10^{-10} M) in *Azospirillum* species. In contrast, the *Pseudomonas putida* thresholds for BA and 4-HB are 5 and 50 μM, respectively (19). *Azospirillum* species can detect potential nutrient sources at much lower concentrations than other soil microorganisms. This ability could contribute to *Azospirillum* survival in oligotrophic soil environments (10, 30).

TABLE 6. Comparison of the chemotactic responses to PCA and CAT by malate- and PCA-grown *A. brasilense* Sp 7

Concn	Mean CI[a]			
	Malate-grown cells		PCA-grown cells	
	PCA	CAT	PCA	CAT
10 mM	1.12 (0.09)	1.90 (0.12)	1.97 (0.28)	2.55 (0.30)
1 mM	1.33 (0.12)	2.21 (0.24)	3.54 (0.59)	2.46 (0.12)
100 μM	1.66 (0.08)	1.72 (0.18)	3.43 (0.36)	2.54 (0.30)
10 μM	1.36 (0.15)	1.48 (0.18)	4.58 (0.66)	2.92 (0.09)
1 μM	1.31* (0.22)	1.29* (0.09)	5.04 (0.25)	3.44 (0.21)
100 nM	1.15 (0.07)	1.10 (0.11)	2.06* (0.13)	2.64* (0.30)
10 nM	ND[b]	ND	0.95 (0.17)	1.26 (0.26)

[a] The CI is calculated as the ratio of cell density in experimental capillaries to that in control capillaries (see Materials and Methods). Results are reported as mean CI with one standard deviation shown in parentheses. Peak response values are boxed, and threshold levels are indicated by asterisks ($P < 0.05$ for all experiments by ANOVA).

[b] ND, not determined.

In addition to its sensitivity, the *Azospirillum* chemotactic response to BA does not require induction by growth on this substrate. Chemotaxis to BA is BA independent, possibly constitutive in *Azospirillum* species, whereas induction is required in *P. putida* (18, 19). However, chemoattraction to 4-HB by *A. lipoferum* was inducible. This was also the case for the chemotaxis of *P. putida* to this substrate (18). Similarities in the *A. lipoferum* dose responses for PCA, CAT, and BA suggest that the same or similar chemoreceptor(s) may be involved in detection of these compounds, but the inducible nature of the 4-HB chemoreceptor implies that it is different. It is noteworthy in this regard that two distinct uptake systems for 4-HB and PCA have previously been reported for *Rhizobium leguminosarum* (36). The 4-HB uptake systems of *A. brasilense* and *A. lipoferum* may also be distinct.

Differences in *Azospirillum* responses to nonaromatic compounds have been attributed previously to their environmental origin (28). *Azospirillum* strains isolated from C_3 and C_4 plants differ in their chemotactic patterns toward sugars and amino acids. This strain selection may be based on variations in the compositions of exudates released from the host plant roots (28), possibly conferring a degree of host specificity for *Azospirillum* strains. In this study, we found that *A. brasilense* responds to much lower levels of aromatic compounds than *A. lipoferum*. Also, despite their consanguinity (15), *A. brasilense* Sp CD was more responsive to all aromatic compounds tested than *A. brasilense* Sp 7. Another interesting difference between the *Azospirillum* species is their response to CAT and PCA when grown on PCA. Production of chemoreceptors for PCA may be induced during growth of *A. lipoferum* on other substrates but repressed during growth on PCA. Conversely, growth on PCA may induce the formation of more PCA chemoreceptors in *A. brasilense*. Clearly, the same aromatic substrate can elicit different chemotactic responses from different *Azospirillum* species. All of these dissimilarities in *Azospirillum* spp. are consistent with previous findings of strain-specific chemotaxis in this genus (28).

Aromatic compounds are catabolized by a wide variety of microorganisms (9, 12, 16, 17, 25, 27, 31) and are common in the soil and rhizosphere, where *Azospirillum* species are frequently abundant (4). On the basis of the ability of *Azospirillum* species to detect these aromatic substrates, at concentrations similar to those they encounter naturally (35), we hypothesize that chemotaxis to aromatic compounds may influence the survival and host selectivity of *Azospirillum* species. Further experiments to test this hypothesis are under way.

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