JOURNAL OF BACTERIOLOGY, Aug. 1985, p. 417–422
0021-9193/85/080417-06$02.00/0
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Vol. 163, No. 2

Chemotaxis to Aromatic and Hydroaromatic Acids: Comparison of *Bradyrhizobium japonicum* and *Rhizobium trifolii*

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Received 20 February 1985/Accepted 30 April 1985

Rhizobia **are bacteria well known for their ability to fix nitrogen in symbiosis with leguminous plants. Members of diverse rhizobial species grow at the expense of hydroaromatic and aromatic compounds commonly found in plant cells and plant litter. Using a quantitative capillary assay to measure chemotaxis, we tested the ability of hydroaromatic acids, selected aromatic acids, and their metabolites to serve as chemoattractants for two distantly related rhizobial species, *Bradyrhizobium japonicum* and *Rhizobium trifolii*. Slow-growing *B. japonicum* I-110 demonstrated positive chemotaxis to shikimate, quinate, protocatechuate, and vanillate; threshold concentrations for the compounds were as low as 10^{-6} M. The dicarboxylic acids succinate and β-ketoadipate, metabolites in the catabolism of many aromatic compounds, were positive chemoattractants with low threshold concentrations as well. Taxis to β-ketoadipate occurred constitutively and, of the tested compounds, β-ketoadipate gave the strongest peak response. Taxis to shikimate or quinate was induced by growth on either substrate but not by growth on protocatechuate or succinate. In contrast, fast-growing *R. trifolii* 2066 was only weakly attracted to quinate and other aromatic and dicarboxylic acids that were strong attractants for *B. japonicum*. The *R. trifolii* strain exhibited positive chemotaxis to shikimate, but the threshold concentration of shikimate required to elicit a response (10^{-4} M) was 2 orders of magnitude higher than that for the *B. japonicum* strain.**

Rhizobia are a group of diverse bacteria that can exist as free-living motile rods or as nonmotile bacteroids which fix nitrogen within legume root nodules. The success of the bacteroid form of a particular rhizobial strain depends in part on the ability of the free-living motile form to compete effectively with other rhizobial strains and with other microorganisms. Motility is likely to be a significant factor in microbial competition for nutrients and in the distribution of rhizobia in the rhizosphere. A motile parental strain of *Rhizobium meliloti* was shown to have a competitive advantage in nodulation over a nonmotile mutant strain (2). The advantage may lie in the ability of a motile strain to undergo chemotaxis, i.e., to sense a chemical concentration gradient and to move up the gradient.

A number of compounds that serve as attractants for diverse strains of rhizobia have been identified. Amino acids and sugars have been most widely studied since they have been identified in legume root exudates (11, 12, 27, 32), and many support the growth of rhizobia (29). Bowra and Dilworth (4) found that a strain of *Rhizobium leguminosarum* was attracted to numerous sugars. Strains of trefoil *Rhizobium* were attracted to sugars (6, 8), and one strain was attracted by a high-molecular-weight glycoprotein present in the root exudate of its host, Birdsfoot trefoil (7, 9). Götz et al. (14) demonstrated the attraction of *R. meliloti* and *Rhizobium lupini* to amino acids and several sugars. Gaworzewska and Carlile (11), using a blind-well assay, showed the attraction of an *R. leguminosarum* strain to the organic acids citrate, gluconate, malate, and succinate as well as to amino acids and sugars. Gitte et al. (12) reported that a cowpea *Rhizobium* strain was attracted to some amino acids, sugars, and three sugar acids found in a host plant root exudate, although the concentrations of these compounds were not specified.

Another class of compounds, aromatic acids, supports the growth of diverse rhizobial species (13, 18, 23, 26). Simple aromatic acids and the hydroaromatic biosynthetic intermediates quinate and shikimate are widely distributed in plants and in decomposing plant material. Genes for the catabolism of protocatechuate via β-ketoadipate (Fig. 1) are widely conserved among members of the family *Rhizobiaceae* (26), and this observation has raised the possibility that aromatic compounds or their metabolites may serve as chemoattractants for members of this family. To test this hypothesis, we chose two quite different representatives of the rhizobia: peritrichously flagellated *Rhizobium trifolii* 2066 and subpolarly flagellated *Bradyrhizobium japonicum* I-110 (10). Shikimate, quinate, selected aromatic compounds, and their metabolites were tested as chemoattractants for the two strains.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *R. trifolii* 2066 and *B. japonicum* I-110 were obtained from H. H. Keyser and L. David Kuykendall, respectively, of the U.S. Department of Agriculture, Beltsville, Md. Both strains were enriched for uniformly motile populations by inoculating them onto swarm plates and reinoculating fresh swarm plates with cells from the outside edge of the swarm (1). Cells were passaged at least three times on swarm plates before use in experiments. The swarm plates contained yeast extract-mannitol medium (YM) (33), with mannitol and yeast extract each reduced to 0.01% (wt/vol), and Noble agar (Difco Laboratories, Detroit, Mich.) at 0.3% (wt/vol).

Swarmed cells were maintained on agar plates containing YM. A colony was inoculated into defined liquid minimal

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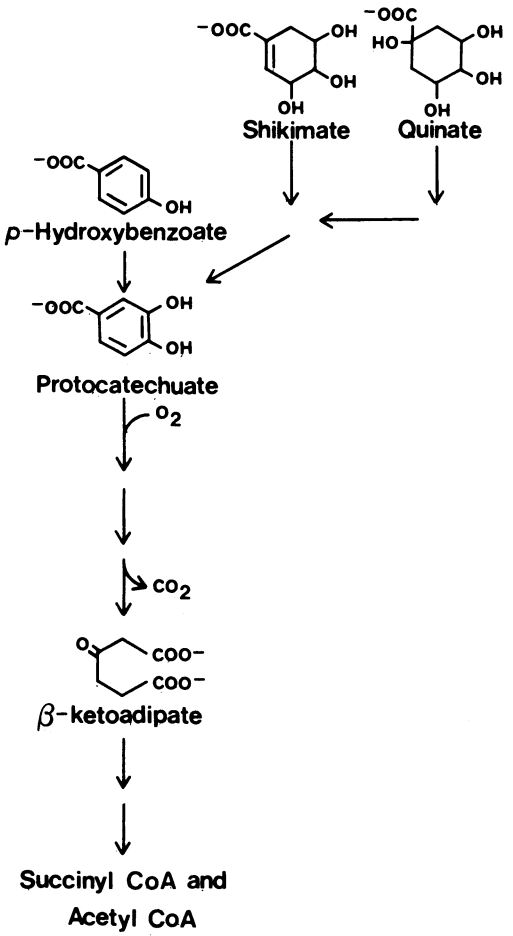

FIG. 1. Metabolic relationships among hydroaromatic and aromatic compounds examined as chemoattractants. Each arrow represents a specific enzymatic step.

medium (25) containing Hutner's metals 44 (5) and biotin at 0.5 μg ml^{-1}; the medium was modified in that it contained a 0.005 M concentration each of KH_2PO_4 and Na_2HPO_4. Shikimate-grown inocula, adjusted to an initial turbidity of 6 Klett units, were used for the growth of *B. japonicum* with vanillate and syringate. The minimal medium for *R. trifolii* contained additional supplements of thiamine hydrochloride at 1 μg ml^{-1} and calcium pantothenate at 2 μg ml^{-1}. With the exception of L-arabinose, which was purchased from Matheson, Coleman and Bell, Cincinnati, Ohio, carbon sources were obtained from Sigma Chemical Co., St. Louis, Mo. The compounds were prepared as 0.5 and 1.0 M concentrated solutions, neutralized with sodium hydroxide to pH 6.8, if necessary, and filter sterilized. Unstable substrates like β-ketoadipate were stored frozen. Previous work demonstrated that some aromatic growth substrates are toxic to rhizobia (26). Consequently, nontoxic substrates like quinate were supplied at 5 mM, and toxic substrates like protocatechuate were supplied at 2 mM. Liquid cultures of 10 ml grown in 50-ml Erlenmeyer flasks were incubated at 30°C on a Gyrotory Environmental Shaker (New Brunswick Scientific Co., Inc., Edison, N.J.) at 240 rpm.

Harvesting of cells. For use in chemotaxis assays, exponentially grown *R. trifolii* cells were harvested at an optical density of 0.12 to 0.21 at 600 nm; *R. trifolii* cells harvested at higher cell concentrations were likely to exhibit reduced chemotaxis. *B. japonicum* cells were harvested at an optical density of 0.18 to 0.34 at 600 nm; *B. japonicum* cells harvested at an optical density below 0.14 were likely to demonstrate a reduced chemotactic response. Cells were centrifuged at 3,000 × *g* for 13 min and rinsed once with chemotaxis buffer (see below). Rinsed cells were gently suspended in chemotaxis buffer to a density of ca. 8×10^7 cells ml^{-1} for *R. trifolii* and 2×10^8 cells ml^{-1} for *B. japonicum*. Sixty to ninety percent of the harvested cells were motile and remained chemotactic for at least 2 h.

The chemotaxis buffer used for both strains was 0.01 M potassium phosphate (pH 7.0) containing 10^{-5} M EDTA. In addition, dithiothreitol at 10^{-4} M was included in the buffer for *B. japonicum* cells. The buffer was prepared with glass-distilled water, autoclaved the day before use, and aerated by being shaken 100 times immediately before chemotaxis assays; filter-sterilized dithiothreitol was added after aeration.

Chemotaxis assays. Chemotaxis and motility were measured at 30°C by the capillary assay of Adler (1). Cells were incubated in a U-shaped chamber created by placing a U-shaped tube between a microscope slide and a cover slip. Motility and background measurements were made by placing the open end of a 1-μl capillary tube containing chemotaxis buffer into the pond of cells. Chemotaxis was measured with a capillary tube containing attractant dissolved in chemotaxis buffer. Cells were incubated in the chambers with the capillary tubes for 30 min at 30°C. At the end of this period, the capillary tubes were rinsed with sterile water, and cells that had entered the capillary tubes were diluted in minimal medium and plated in duplicate onto YM plates. Colonies were counted after incubation for 2 days at 30°C for *R. trifolii* and after incubation for 5 days at 30°C for *B. japonicum*.

Chemotaxis assays were performed in duplicate or triplicate and repeated at least once. Concentrations of attractant from 10^{-6} M to 10^{-1} M were tested. The averages of representative experiments done in duplicate or triplicate on a single day are reported in this paper. All experiments included a capillary tube containing buffer with no attractant and a positive control. The definitions of Mesibov and Adler (22) for threshold concentration, peak response, peak concentration, and concentration-response curve were used here. Threshold values were determined by using linear regression to extrapolate to the background base line a double-log plot of the concentration-response curve (22). The relative response is the ratio of the number of cells that entered a capillary tube containing attractant to the number of cells that entered a capillary tube not containing attractant.

RESULTS

Optimization of motility and chemotaxis in *R. trifolii*. Although 10^{-5} M EDTA was included in the chemotaxis buffer, essentially the same results were obtained when it was omitted. A 10-fold-higher concentration of EDTA, used by other workers (4, 7), inhibited motility and chemotaxis in our system, possibly because the levels of chelators were not the same in the different growth media. Motility did not vary significantly across the pH range 6.0 to 7.5; pH 7.0 was used for assays.

The accumulation of cells in a capillary tube in response to 5×10^{-4} M arabinose at 30°C increased linearly with time up to 45 min. After 45 min, the number of bacteria in the

capillary tube fell off. A similar drop in the response to amino acids after 30 min was reported with *Bacillus subtilis* (31). A 30-min incubation period was used in our assays.

The number of cells migrating towards 5×10^{-4} M arabinose and towards buffer containing no attractant increased linearly as the concentration of cells in the pond was increased from 3×10^7 to 1.5×10^8 cells ml^{-1}. The standard deviation for the attraction of cells into a capillary tube containing 5×10^{-4} M arabinose was 15% in assays on nine different days; the standard deviation for experiments performed in triplicate on a single day was 6% or less. The number of cells entering the capillary tube ranged from 500 to 2,000. Although the absolute numbers of cells entering the capillary tube and the relative response to a given compound varied on different days, threshold values were constant even when the cells had reduced motility. Other workers have commented on the variability of the chemotactic response in *Rhizobium* spp. (11, 14).

Arabinose at 5×10^{-4} M was used as a positive standard in chemotaxis asays with *R. trifolii*. Yeast extract of 0.1% served as a positive standard in the initial characterization of arabinose chemotaxis. Götz et al. (14) found L-proline to be an effective attractant for *R. meliloti* and *R. lupini* and used it as a positive standard in chemotaxis assays. Proline proved to be only a weak attractant for proline- or quinate-grown *R. trifolii* 2066 under our conditions. A concentration-response curve for arabinose showed the peak to be 5×10^{-4} M, with a mean of 70,000 cells entering the capillary tube against a background of 1,000 cells. The threshold for attraction was 2×10^{-7} M. When arabinose at 5×10^{-4} M was present in the pool with the cells and in the capillary tube, no stimulation of motility over the background accumulation occurred. Thus, the response of the cells to arabinose in the standard capillary assay was a response to a chemical gradient. When cells were grown on quinate or related compounds, the chemotactic response to arabinose was similar to the response of cells grown at the expense of 5 mM arabinose.

Chemotaxis of *R. trifolii* to shikimate. *R. trifolii* was positively attracted to shikimate; quinate was a poor attractant (Table 1). The peak response to shikimate occurred at 0.1 M or higher, and the threshold was 3×10^{-4} M. This contrasts with a threshold of 2×10^{-7} M for arabinose. The response to shikimate was not observed when 50 mM shikimate was present in the pool with the cells and in the capillary tube. Thus, the response was to a concentration gradient. The observed chemotaxis to shikimate was not merely a response to the sodium ions used to neutralize the acid (Table 1). The supplier reported that the chemical purity of the shikimate was determined to be 99.8% by gas chromatography, so we cannot exclude the possibility that the observed chemotaxis was in response to a contaminating chemical with a threshold of 6×10^{-7} (assuming that the putative contaminant and shikimate have similar molecular weights). *R. trifolii* 2066 does not grow at the expense of shikimate, whereas it does grow with quinate as the sole carbon source (26). This strain appears to lack the expression of a shikimate uptake system or the first catabolic enzyme, shikimate dehydrogenase (Fig. 1). Thus, the metabolism of shikimate is not necessary for chemotaxis to occur. Chemotaxis of *R. trifolii* to shikimate was expressed at low to moderate levels in arabinose-grown cells and at higher levels in quinate- or protocatechuate-grown cells. A mutant strain, 2066-S1, which grows at the expense of shikimate, did not show any higher response to shikimate when it was grown at the expense of shikimate rather than quinate.

TABLE 1. Chemotactic responses of *R. trifolii* 2066[a]

Compound	Doubling time (h)	Threshold concn (M)	Peak concn (M)	Peak response[b]
L-Arabinose	4	2×10^{-7}	5×10^{-4}	70,000
Gallate	NG[c]	1×10^{-4}	1×10^{-2}	20,400
β-Ketoadipate	NG	1×10^{-5}	1×10^{-2}	1,500
Quinate	9	2×10^{-3}	5×10^{-2}	3,100
Shikimate	7[d]	3×10^{-4}	1×10^{-1}	40,000
Sodium chloride	NG	1×10^{-2}	1×10^{-1}	1,100
Succinate	3[e]	2×10^{-7}	1×10^{-4}	2,900

[a] Cell concentrations were 8×10^7 cells ml^{-1}; compounds were tested at concentrations ranging from 10^{-6} to 10^{-1} M. Cells were grown on quinate.

[b] Reported as the number of cells that entered a capillary tube containing the peak concentration of attractant minus the number of cells entering a capillary tube containing no attractant.

[c] NG, Poor or no growth in shake culture.

[d] Variant strain 2066-S1 cells, which grow at the expense of shikimate, have a doubling time of 7 h with this substrate. The threshold and peak concentrations for shikimate were the same for the variant strain grown with shikimate as for the quinate-grown wild type.

[e] Succinate-grown cells were largely immotile.

When cells were grown at the expense of the compounds being tested as attractants, protocatechuate, *p*-coumarate, and *p*-hydroxybenzoate were weaker attractants than quinate. *R. trifolii* cells grown at the expense of quinate displayed a moderate attraction to gallate but only a weak attraction of succinate and β-ketoadipate (Table 1). The peak (10^{-4} M) and threshold (10^{-7} M) concentrations for succinate were low, however. We were unable to test for the induction of succinate chemotaxis because when cells were grown at the expense of 2 mM succinate they were poorly motile. Morphological changes associated with the growth of *R. trifolii* cells in the presence of succinate have been reported by others (30).

Optimization of chemotactic assays with *B. japonicum* cells. The rationale for including dithiothreitol at 10^{-4} M as well as EDTA at 10^{-5} M in chemotaxis buffers was to reduce interference from heavy metals, which have been shown to inhibit motility (4). Dithiothreitol appeared to reduce the standard deviation, and it had no effect on threshold values. As was the case with *R. trifolii*, the use of a 10-fold-higher concentration of EDTA inhibited motility and chemotaxis. Motility and chemotaxis did not vary significantly across the pH range 6.0 to 7.5; pH 7.0 was used for all experiments.

The accumulation of cells in a capillary tube in response to 10^{-3} M shikimate at 30°C increased linearly with time up to 45 min. The standard period of incubation was 30 min. The number of cells that migrated towards 10^{-3} M shikimate and towards buffer containing no attractant increased linearly as the concentration of cells in the pond was increased from 1.2×10^8 to 4.5×10^8 cells ml^{-1}. Below 10^8 cells ml^{-1}, the standard deviation was 50% in assays on six different days; on some days no chemotactic response was observed. With a cell concentration above 1.25×10^8 cells ml^{-1}, the standard deviation in assays on six different days was 20%. With a cell concentration of 2×10^8 cell ml^{-1}, the background accumulation of cells in a capillary tube ranged from 3,300 to 13,300 cells on different days. The number of cells that entered a capillary tube in response to 10^{-3} M shikimate ranged from 87,000 to 300,000 when the cell concentration for the assays was 2×10^{-8} cells ml^{-1}. As with *R. trifolii*, the number of cells which accumulated at the peak and thus the relative responses were variable, but the threshold values

TABLE 2. Chemotactic responses of *B. japonicum* I-110[a]

Compound	Doubling time (h)	Threshold concn (M)	Peak concn (M)	Peak response[b]
Adipate	12	8×10^{-7}	10^{-3}	110,500
L-Arabinose[c,d]	30		10^{-3}	9,500
β-Ketoadipate[c]	ND[e]	3×10^{-7}	10^{-3}	395,500
p-Coumarate[c]	ND	6×10^{-8}	10^{-2}	67,500
p-Hydroxybenzoate[c]	ND	6×10^{-7}	10^{-2}	31,250
Protocatechuate	30	1×10^{-6}	10^{-2}	95,000
Quinate	15	3×10^{-6}	10^{-3}	151,000
Salicylate[c]	NG[f]	1×10^{-6}	10^{-3}	21,250
Shikimate	12	7×10^{-7}	10^{-3}	135,000
Sodium chloride		No response		
Succinate	12	1×10^{-6}	10^{-3}	115,000
Vanillate	35	3×10^{-6}	10^{-2}	130,500

[a] Assays were conducted with 2×10^8 cells ml^{-1}; compounds were tested at concentrations ranging from 10^{-6} to 10^{-1} M. Except as noted, cells were grown on the compounds tested as chemoattractants.
[b] See Table 1, footnote *b*.
[c] Shikimate-grown cells.
[d] Arabinose-grown cells were not attracted to arabinose.
[e] ND, Not determined.
[f] NG, Poor or no growth.

were reproducible. Depending on the conditions of growth, 10^{-3} M shikimate, 10^{-3} M succinate, 0.1% yeast extract, or combinations thereof were used as positive standards in chemotaxis assays.

Chemotaxis of *B. japonicum* to shikimate and other organic acids. When *B. japonicum* cells were grown at the expense of the compounds being tested as chemoattractants, they were attracted to protocatechuate, quinate, shikimate, succinate, and vanillate (Table 2). In addition, cells grown at the expense of shikimate were strongly attracted to β-ketoadipate (Table 2). Adipate, a dicarboxylic acid metabolized via β-ketoadipyl coenzyme A (17), supported the growth of and served as a chemoattractant for *B. japonicum* (Table 2).

The threshold concentrations for the weak acids ranged from a low of 3×10^{-7} M for β-ketoadipate to a high of 3×10^{-6} M for quinate. In contrast to the *R. trifolii* strain, the *B. japonicum* strain was not significantly attracted to arabinose (Table 2). No response over the background occurred when 10^{-3} M shikimate, quinate, succinate, or β-ketoadipate was present in the cell suspension and in the capillary tube. Thus, the attraction of cells to these compounds was dependent on a concentration gradient. The possibility that *B. japonicum* was attracted to the sodium ions used in neutralizing the acids was examined. Filter-sterilized sodium chloride at 10^{-5}, 10^{-4}, 10^{-3}, and 10^{-2} M failed to stimulate the motility of the cells over the background under conditions in which the relative response to shikimate was 20.

Some other compounds which support the growth of *B. japonicum* at low concentrations (26) were tested as chemoattractants. *p*-Coumarate and *p*-hydroxybenzoate were positive chemoattractants (Table 2). Gallate, a chemoattractant for *R. trifolii* (Table 1), did not elicit a positive chemotactic response in *B. japonicum*. Salicylate, which does not support the growth of *B. japonicum* I-110 (26), was a positive chemoattractant (Table 2).

Regulation of chemotaxis towards organic acids in *B. japonicum*. Chemotaxis to β-ketoadipate (Fig. 2) and to succinate was expressed constitutively in *B. japonicum* I-110. Chemotaxis to shikimate (Fig. 3), quinate, and protocatechuate was expressed at high levels when the cells were grown on the homologous substrates but poorly when the cells were grown at the expense of succinate. Furthermore, chemotaxis to shikimate (Fig. 3) and quinate was expressed when the cells were grown at the expense of quinate but not when they were grown at the expense of protocatechuate. Protocatechuate-grown cells, which failed to respond to shikimate and quinate, had high levels of

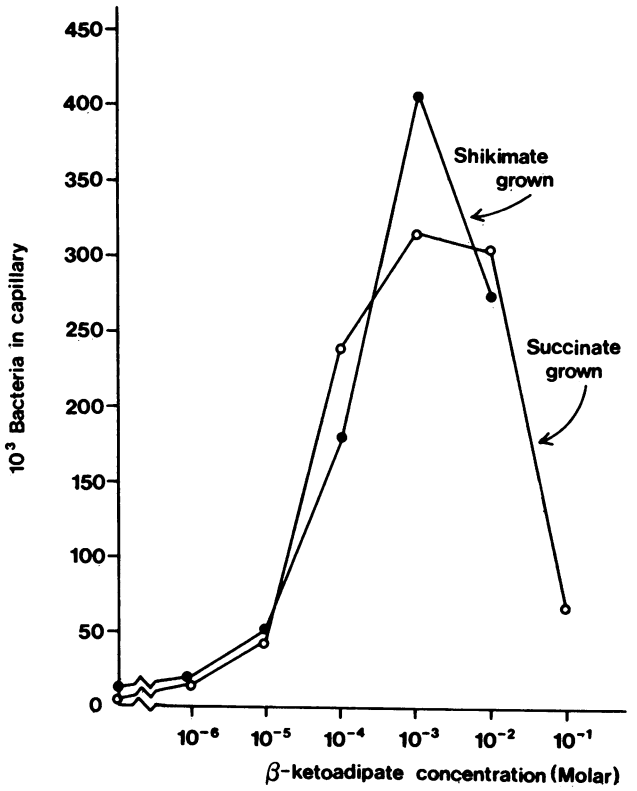

FIG. 2. Concentration-response curves for β-ketoadipate chemotaxis in *B. japonicum* I-110.

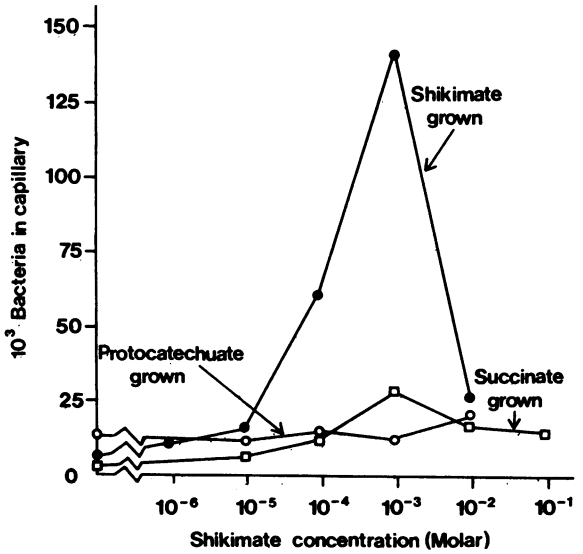

FIG. 3. Concentration-response curves for shikimate chemotaxis in *B. japonicum* I-110.

chemotaxis to protocatechuate, succinate, and β-ketoadipate.

DISCUSSION

B. japonicum I-110 is considered to be a slow-growing rhizobium with a doubling time of 6 h or more on YM; the doubling time of fast-growing *R. trifolii* 2066 on YM is 2 to 4 h. It is clear that the slow metabolism of *B. japonicum* is not reflected in sluggish chemotaxis. The unequivocal ability of *B. japonicum* to respond chemotactically to hydroaromatic, aromatic, and dicarboxylic acids indicates that these classes of compounds play a role in the ecology of the organism. The diversity of plant phenolics affords many opportunities for specific plant-bacteria interactions. A number of these are recognized as plant defense mechanisms (3, 28), but plant phenolics also might serve as developmental signals or chemoattractants in the establishment of specific symbiotic interactions.

It is noteworthy that the β-ketoadipate chemotactic system is formed in the absence of an inducer in *B. japonicum*. Several rhizobial species exhibit constitutive chemotactic responses to carbohydrates that serve as nutrients (4, 11, 14). In contrast, chemotactic systems for non-nitrogenous compounds are inducible in coliform bacteria (21).

β-Ketoadipate, known to accumulate during the catabolism of many aromatic compounds (20, 24), may serve as a signal attractant that swiftly brings *B. japonicum* to sites where aromatic metabolism by other organisms is under way. The possibility that β-ketoadipate contributes to the formation of catabolic consortia is raised by the fact that the compound acts as a chemoattractant for *Pseudomonas putida* (19), another microbe commonly found in soil and aquatic environments.

R. trifolii, capable of growth with a number of aromatic compounds, appears to be less well adapted to respond behaviorally to the compounds that stimulate chemotaxis in *B. japonicum*. We cannot rule out the possibility that *R. trifolii* 2066 is attracted to these aromatic compounds when they are covalently bound to other aromatic compounds or sugars.

What role such different responses may play in the attraction of rhizobia to the vicinity of plant roots or to specific sites on a root remains an open question. Gulash et al. (15) found that *R. meliloti* was attracted to localized sites on legume roots. This observation demonstrates that microenvironments exist in the rhizosphere. Studies of total root exudates may reveal little about significant chemical gradients occurring at localized sites. Even a compound having a high threshold for chemotaxis, as shikimate does for *R. trifolii*, may prove to be significant in microrange bacteria-plant interactions.

The physiological control of chemotaxis can be quite indirect, as demonstrated by the induction of benzoate chemotaxis by β-ketoadipate, a metabolite seven enzymatic steps removed, in *P. putida* (16). Shikimate, quinate, protocatechuate, and other aromatic compounds may attract cells by virtue of their conversion to metabolites that interact with one or more chemoreceptors. The metabolic block that prevents the metabolism of shikimate in *R. trifolii* 2066 suggests that shikimate directly elicits chemotaxis in this strain. Mutant rhizobial strains blocked in the conversion of chemoattractants should help to elucidate control mechanisms and to define the minimum number of primary chemoattractants represented among the surveyed compounds.

ACKNOWLEDGMENTS

We thank C. S. Harwood for advice and support. We express appreciation to Anne Harrison for preparation of the manuscript. This research was funded by Celanese Research Co.

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