Chemotaxis of *Erwinia amylovora*

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ABSTRACT

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Chemotaxis of *Erwinia amylovora* is temperature- and pH-dependent with an optimum temperature range of 20–28 C and pH 6–8. An incubation period of 30 min and a cell population not greater than 4×10^7 cells per milliliter are optimal for chemotaxis studies. A medium consisting of 10^{-3} M ethylene diaminetetraacetic acid, 10^{-3} M mannitol, 10^{-2} M $MgCl_2$ and 10^{-2} M potassium phosphate buffer at pH 7 was established for assays. Using these assay conditions, *E. amylovora* exhibits positive chemotaxis to apple nectar, to the organic acid fraction of apple nectar, to one amino acid (aspartate), to the organic acids fumarate, malate, maleate, malonate, oxaloacetate, and succinate, but not to any of the sugars tested. All attractants are dicarboxylic acids and responses of *E. amylovora* are uniformly inhibited by malate, suggesting a single chemoreceptor site for all the attractants. The chemoattractant response pattern of our strain and that of American Type Culture Collection strain 19382 of *E. amylovora* was identical.

Additional key words: fire blight, apple, motility, bacterial movement.

Motility may be a directed motion, and when it is in response to a chemical gradient it is called chemotaxis. Chemotaxis, positive or negative, has been demonstrated in a variety of bacterial genera (18). Among plant pathogenic bacteria, chemotaxis studies have been limited. *Xanthomonas oryzae* was more attracted to exudates from susceptible than from resistant rice plants (6), and *Pseudomonas lachrymans* to extracts from both susceptible and resistant cucumber plants (5). It was suggested that chemotaxis in *P. phaseolicola* is important in the location of infection courts (12). However, factors affecting motility and chemotaxis were not established before any chemotactic response of the above mentioned bacteria was studied. Furthermore, the responses of these bacteria to pure compounds at different concentrations were not tested.

Additional studies in chemotaxis of plant pathogenic bacteria are needed if generalizations concerning bacterial chemotaxis are to be made. Intensive studies have been limited to *Escherichia coli, Salmonella typhimurium,* and *Bacillus subtilis* (7). *Erwinia amylovora* (Burr.) Winslow et al, the widely studied causal organism of fire blight, should serve as a good model to study chemotaxis of plant pathogenic bacteria. Such studies may eventually aid in the elucidation of the role of chemotaxis in plant pathogenesis and may be used as a basis to explore the employment of attractants or repellents to deter the infection process.

This paper reports the factors affecting chemotaxis of *E. amylovora* and the bacterium's response to amino acids, certain sugars, and some organic acids. Factors affecting motility of *E. amylovora* have been reported (15).

MATERIALS AND METHODS

Bacterial strain and growth conditions. The bacterial strain of *E. amylovora* (A_1) used was a single-cell isolate obtained from an infected apple tree, the same strain used in motility studies (15). *Erwinia amylovora* was grown on Miller and Schroth medium (10) modified by leaving out the selective agents (MMS): sodium taurocholate, Tergitol anionic 7, nitriloacetic acid, bromothymol blue, neutral red, cycloheximide, and thallium nitrate and agar. Cultures were incubated in a shaker bath at 120–140 oscillations per minute at 23 C unless otherwise specified.

Factors affecting chemotaxis. A modified Adler's capillary technique described earlier (15) was used to assess the effects of different factors affecting chemotaxis. A capillary tube containing a test chemical was inserted into a bacterial suspension. Bacteria accumulate in the capillary if they are attracted to the chemical. Temperature effects were studied by performing chemotaxis assays on slide warmers adjusted to the assay temperature and placed in a refrigerated room. The effects of pH, incubation time, $MgCl_2$, chemotaxis medium, and bacterial concentration on chemotaxis also were investigated by varying each parameter.

Apple nectar extract served as the natural chemoattractant. Nectar extracts were obtained from flowers at full bloom of cultivars Jonathan and Golden Delicious at the University of Illinois apple orchard. Glass distilled water (10 μl) was deposited on flower nectaries exposed by manually removing other flower parts for 10–15 sec and then removed with an Eppendorf pipet. The nectar extracts were filter sterilized and either lyophilized or stored at −20 C. One milliliter of the extract was obtained from 130 flowers and it weighed 3.1 mg after freeze drying. Fractionation of nectar extract into basic, neutral and amino acid, and organic acid fractions was performed with an anionic resin (Dowex 2-X8-chloride form, 74–38 μm [200-400 mesh]) and a cationic exchanger (Dowex 50W-X8-hydrogen form, 74–38 μm). A vial of the freeze-dried nectar extract containing 3.1 mg was rehydrated with 5 ml of glass distilled water and added to 5 ml of Dowex-X8 (wet volume), mixed, filtered, and the resin washed with 0.1 N HCl and refiltered. Dowex 50W-X8 was added to the latter filtrate, and the same process was repeated as above. All filtrates were evaporated to dryness, dissolved in 10 ml of the chemotaxis medium (equivalent to a 10^{-1} dilution of the crude nectar extract) consisting of 10^{-3} M ethylene diaminetetraacetic acid (EDTA), 10^{-3} M mannitol, 10^{-2} M $MgCl_2$ and 10^{-2} M potassium phosphate buffer at pH 7. The pH was adjusted to 7 with KOH and the solutions assayed by the capillary technique.

Chemotaxis assays of amino acids, sugars, and organic acids. The capillary tube assay previously described was used. The different test chemicals at 10^{-1}–10^{-7} M (depending upon solubility) were dissolved in the chemotaxis medium described previously. The solutions were adjusted to pH 7 with KOH as necessary.

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Assays were run for 30 min by using a bacterial population containing 8×10^6 cells per milliliter suspended in the chemotaxis medium. In assays of sugar compounds, the bacteria were grown on Modified Miller Schroth Medium (MMS) with a 10^{-2}M concentration of the sugar being assayed. Cysteine solutions were prepared just before assay.

All sugars used were D-form and all amino acids L-form (Sigma Chemical Co., St. Louis, MO 63178). All organic acids were all reagent grade obtained from various commercial sources.

Terminology. Terms commonly used to describe chemotaxis responses are adopted (1, 9). A concentration response curve is a plot of responses versus the logarithm of concentration in the capillary. Peak concentration is defined as the concentration that causes the greatest accumulation of bacteria in a series of dilutions. Peak response is the number of bacteria that accumulate in the capillary containing the peak concentration. Threshold is the concentration which gives a detectable increase over the blank or control value (response to zero concentration of the compound) plus the standard deviation for replicate determination for the value. The threshold may be extrapolated from a concentration response curve on a double logarithmic plot. The blank value is the bacterial accumulation in the absence of an attractant. Relative response is the ratio of the number of bacteria per capillary to that of blank value (11). Any chemical with a relative response less than five at the peak concentration is considered to be a weak attractant in this study. Threshold values of weak and non-attractants were not computed.

RESULTS

Factors affecting chemotaxis. *Chemotaxis medium composition.* Both an energy source and EDTA are essential for chemotaxis of *E. amylovora* toward nectar extract. The use of a chemotaxis medium consisting of 10^{-3}M $(NH_4)_2SO_4$, 10^{-5}M $MgCl_2$, 10^{-3}M mannitol, 10^{-3}M EDTA, and 10^{-2}M potassium phosphate buffer at pH 7 resulted in an accumulation of 6,400 bacteria per capillary. The removal of EDTA or mannitol, but not $(NH_2)_4SO_4$ and $MgCl_2$ from the chemotaxis medium resulted in statistically fewer bacteria per capillary. Increasing concentration of $MgCl_2$ to 10^{-2}M increased the accumulation to 30,000 bacteria per capillary. Based on the above results, the chemotaxis medium used in our studies consisted of 10^{-2}M potassium phosphate buffer (pH 7), 10^{-3}M EDTA, 10^{-3}M mannitol and 10^{-2}M $MgCl_2$.

Effect of incubation period. Accumulation of bacteria attracted by 10^{-1} dilution of nectar extract in the capillary reached a maximum after 30 min. The responses obtained after 30 min did not vary significantly ($P = 0.05$) from responses at 45, 60, 75, and 90 min. Accumulation in the absence of an attractant increased linearly with time to about 830 bacteria in the capillary after 90 min of incubation. Therefore, an incubation period of 30 min was adopted.

Effect of pH. Chemotaxis towards 10^{-1} dilution of nectar extract was unaffected within a range of pH 6–8. Values of pH 4 and 10 caused an almost complete inhibition of chemotaxis. Response at pH 5 and 9 also were significantly ($P = 0.05$) lower than those at pH 6–8.

TABLE 1. Response of *Erwinia amylovora* to the different fractions of nectar extract of apple cultivar Golden Delicious

Fraction[a]	Bacteria per capillary[b]
No attractant	175 w[c]
Neutral and basic	945 w
Amino acid[d]	1,385 w
Organic acid[d]	16,915 x
Unfractionated nectar extract	25,025 y

[a] Fractionated by ion exchange chromatography.
[b] Assay run at 23 C for 30 min with 8×10^6 cells per milliliter outside the capillary.
[c] Means followed by the same letters are not significantly different, $P = 0.05$, according to Fisher's least significant difference test.
[d] Adjusted to pH 7 with KOH. Accumulation in capillary containing 1 M KCl was 240; thus, the values reported are due to the acids themselves.

Effect of temperature. Chemotaxis toward the 10^{-1} dilution of nectar extract was insensitive to incubation temperature within the range of 20–28 C. Responses at 18 and 30 C were significantly ($P = 0.05$) lower than those at 20–28 C, and lowest at 4 and 33 C. The assay temperature used in subsequent experiments was 23 C.

Effect of bacterial concentration. The accumulation of bacteria within the capillary containing 10^{-1} dilution of nectar extract increased linearly with increasing bacterial concentration outside the capillary up to about 4×10^7 cells per milliliter. At higher cell concentrations no additional accumulation occurred; a plateau was reached. Accumulation in the absence of an attractant increased linearly with increasing bacterial concentration up to 7.9×10^7 cells per milliliter, the highest concentration tested. The bacterial population used in all other experiments was 8×10^6 cells per milliliter.

Chemotactic response toward different compounds. *Taxis toward nectar extract and its components.* The response of *E. amylovora* to nectar extracts of Jonathan and Golden Delicious apple cultivars were identical (Fig. 1). Peak concentration occurred at the undiluted form of the nectar extracts and the threshold was $< 1 \times 10^{-6}$ dilution.

Chemotaxis toward the different Dowex fractions of nectar extract of Golden Delicious varied (Table 1). The organic acid fraction was the best attractant among the different fractions, but it was not as good an attractant as the unfractionated nectar extract. Response to the neutral and basic fraction and the amino acid fraction was significantly ($P = 0.05$) lower than the organic acid fraction and not significantly different from the control. No fractionation was performed on the Jonathan nectar extract.

Taxis toward amino acids. Among the amino acids that stimulated a positive significant ($P = 0.05$) response, only aspartate induced high response (13,520 bacteria) at peak concentration compared to 210–1,475 bacteria for the others. The relative responses at peak concentrations was 80.5 for aspartate and 1.5–4.8 for the other amino acids (Table 2). The threshold value for aspartate is less than 10^{-7}M, the lowest concentration tested (Fig. 2). No significant responses ($P = 0.05$) were obtained with alanine, asparagine, L-carbamyl aspartate, glutamine, glycine, hydroxyproline, isoleucine, and lysine.

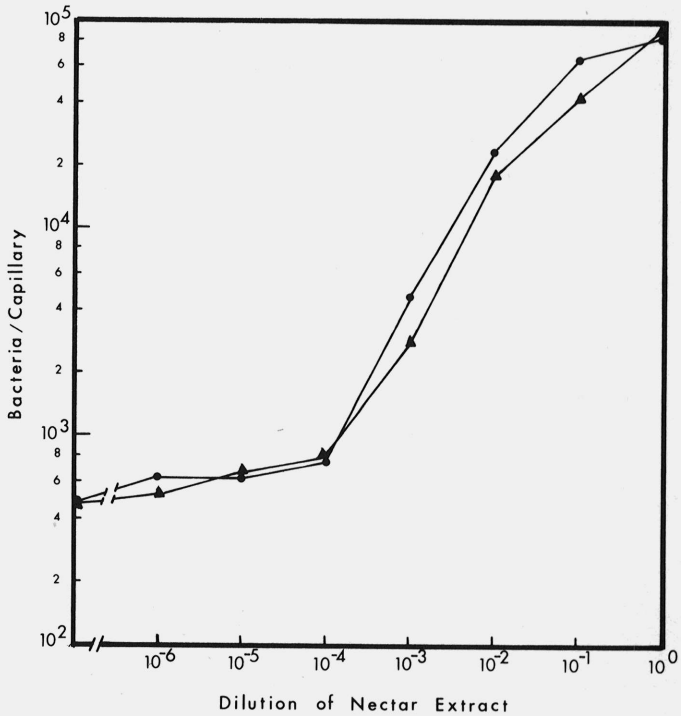

Fig. 1. Concentration response curves for *Erwinia amylovora* attraction to Golden Delicious (•) and Jonathan (▲) apple nectar extracts. Undiluted extract (10°) contains 3.1 mg freeze-dried nectar rehydrated in 1 ml of chemotaxis medium. Assays were run for 30 min at 23 C with 8×10^6 cells per milliliter outside the capillary.

Taxis toward organic acids. Erwinia amylovora demonstrated positive chemotaxis toward eight of 15 organic acids tested (Table 3). Galacturonate and tartarate are weak attractants with peak relative responses of only 3.4 and 2.3, respectively. The peak relative responses of maleate was 5.1, a much lower value than those of the other organic acid attractants. It also has a low threshold value of 8×10^{-3} M (Table 3 and Fig. 2). Fumarate has high peak and relative responses, but a low threshold of 2×10^{-3} M (Table 3 and Fig. 2). Oxaloacetate, succinate, malate, and malonate are good attractants by all criteria (Table 3 and Figs. 2 and 3). All the attractants had peak concentrations of 10^{-1} M except malate and tartarate at 10^{-3} M (Table 3). However, reduced motility of *E. amylovora* was observed in the presence of 10^{-1} M concentrations of the above compounds.

Concentrations of citrate, isocitrate, oxalate, lactate, pyruvate, α-ketoglutarate and *cis*-aconitate at 10^{-1} M to 10^{-7} M concentration did not induce significant ($P = 0.05$) positive responses of *E. amylovora*. Induction of chemotaxis toward citrate was attempted by adding 10^{-2} M citrate to the growth medium. However, poorly motile cells resulted. At 10^{-3} M, cells were motile but not as vigorous as those grown in the absence of citrate. No additional assays were done on citrate.

Taxis toward sugars. Sucrose, glucose, and fructose which are present in nectar extract did not elicit any chemotactic response from *E. amylovora*. The same was true with galactose, mannitol, sorbitol, ribose, lactose, and raffinose. In this set of assays, glutamine previously established to be an nonattractant (authors' *unpublished*), was used as energy source.

Inhibition of chemotaxis by other attractants. Malate, an attractant, when present at 10^{-3} M (peak concentration) in the capillaries containing other attractants and in the cell suspension, inhibited chemotaxis toward all the other attractants tested. Responses to malate and galacturonate were completely inhibited while those to fumarate, succinate, oxalacetate, malonate, aspartate, and nectar extract were reduced by 91, 93, 94, 95, 94, and 98%, respectively.

Comparison of chemotaxis between two strains of *E. amylovora*. The pattern of response of our strain used in this study and ATTC strain 19382 to two sugars, three organic acids, and two amino acids was similar. Neither strain responded to asparagine, fructose, glucose, or α-ketoglutarate. Strong taxis was observed in both strains toward aspartate, malate, nectar extract, and succinate.

TABLE 2. Comparison of response of *Erwinia amylovora* to certain amino acids

Amino acid[a]	Peak response: Concentration (M)	Peak response: Number of bacteria per capillary[b]	Relative response[c]
Valine	10^{-2}	740	2.7
Leucine	10^{-2}	622	2.9
Arginine	10^{-2}	510	3.8
Histidine	10^{-6}	1,330	2.2
Tryptophan	10^{-6}	210	1.7
Tyrosine	10^{-3}	280	1.5
Proline	10^{-4}	650	1.8
Phosphoserine	10^{-5}	1,383	4.8
Cysteine	10^{-4}	475	2.5
Cystine	10^{-3}	1,475	3.5
Methionine	10^{-3}	740	1.6
Glutamate	10^{-1}	545	4.4
Aspartate	10^{-1}	13,520	80.5

[a] Amino acids which gave positive significant responses. Highest concentration tested was 10^{-1} M except for arginine and tryptophan at 10^{-2} M, tyrosine and cystine at 10^{-3} M and nectar extract at 10^{-1} dilution.
[b] Blank values (no attractant in the capillary) were subtracted. Assays were run for 30 min at 23 C with 8×10^6 cells per milliliter outside the capillary.
[c] Ratio of peak response to that of blank value.

TABLE 3. Comparison of responses of *Erwinia amylovora* to selected organic acids

Organic acid[a]	Peak response: Concentration (M)	Peak response: Number of bacteria per capillary[b]	Relative response[c]	Threshold
DL-Tartarate	10^{-3}	575	2.3	...
D-Galacturona	10^{-1}	708	3.4	...
Maleate	10^{-1}	2,230	5.1	8×10^{-3}
DL-Malate	10^{-3}	25,693	161.6	8×10^{-7}
Fumarate	10^{-1}	24,915	56.4	2×10^{-3}
Succinate	10^{-1}	33,295	334.0	2×10^{-7}
Malonate	10^{-1}	34,164	214.5	3×10^{-5}
Oxaloacetate	10^{-1}	20,720	57.0	$<1 \times 10^{-7}$

[a] Organic acids that showed significant responses.
[b] Blank values (no attractant in the capillary) were subtracted. Assays were run for 30 min at 23 C with 8×10^6 cells per milliliter outside the capillary.
[c] Ratio of number of bacteria per capillary of peak response to that of the blank value.

DISCUSSION

Erwinia amylovora exhibits chemotaxis. Medium components and environmental factors influence its response. An energy source, a chelating agent, and $MgCl_2$ were found to be important components of the chemotaxis medium. Similar chemical constituents also are necessary in the *Bacillus subtilis* chemotaxis medium (13). The exact role of these components is not known, but is believed due to their influence on motility (13,15).

Environmental factors, including temperature and pH, affect the chemotactic response of *E. amylovora*. At 28–35 C or at pH 9, responses were reduced but motility was unaffected (authors' *unpublished*, and [15]). This differential response suggests that there is a process in chemotaxis but not in motility that is temperature- and pH-sensitive under the above conditions.

Erwinia amylovora is attracted strongly to aspartate, and to several Kreb's cycle organic acids, but to none of the sugars tested. This pattern of responses differs from those of other bacteria (3,8,13,17) which respond positively to many sugars and several amino acids. The lack of response to sugars was not due to lack of

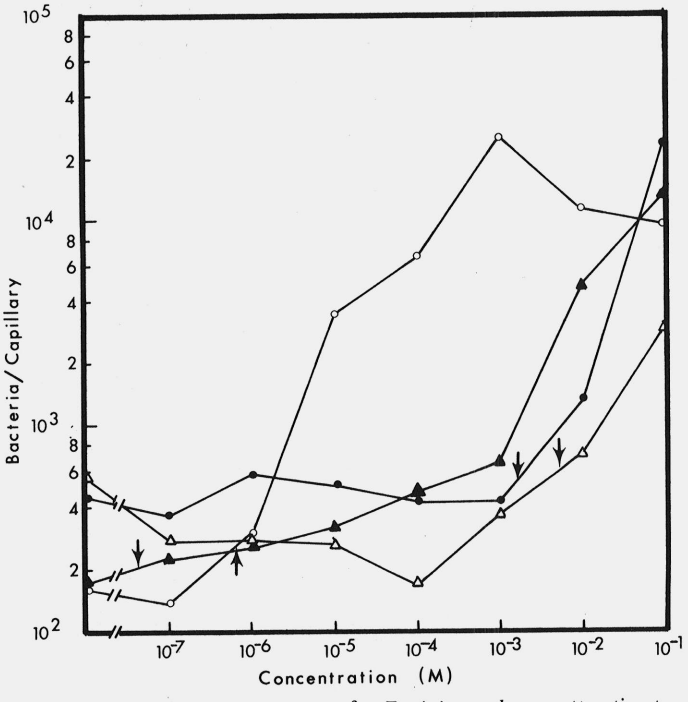

Fig. 2. Concentration response curves for *Erwinia amylovora* attraction to asparate (▲), fumarate (•), malate (O), maleate (Δ). Arrows denote response at threshold (accumulation in value in the absence of attractant plus standard deviation; 30 for fumarate, 67 for malate, and 141 for maleate) based on linear portions of the curves. Assays were run for 30 min at 23 C with 8×10^6 cells per milliliter outside the capillary.

induction since attempts to induce chemoreceptors with appropriate sugar compounds were unsuccessful. Chemoreceptors for sugars and for citrate in some bacterial species are inducible (3,11). *Erwinia amylovora* did not exhibit taxis to citrate, and induction of taxis was not possible because the cells were poorly motile when 10^{-2}M or higher concentrations of citrate were added to the growth medium. Citrate may serve as a catabolite repressor to flagella synthesis in *E. amylovora* resulting in poorly motile cells possibly due to fewer or shorter flagella. Such an effect has been reported with glucose on the flagella synthesis of *E. coli* (4).

The amino and organic acids with peak relative responses of less than five may not be weak attractants, but nonattractants. The presence of minute quantities of a strong attractant in nonattractants could result in a weak positive response (8). Although the strong attractants were not purified, relative responses were 50 or more times higher than responses of weak attractants. Responses toward strong attractants, therefore, are likely due to the compounds themselves.

Erwinia amylovora attractants studied appear to be detected by only one chemoreceptor site. This site appears highly specific. The substitution of a hydroxyl group for a hydrogen on the C_3 of malate, changes malate, a strong attractant, to tartarate, a weak attractant. The substitution of an amide group for a carboxyl group changes aspartate, a strong attractant to asparagine, which is not an attractant. The addition of a carbon atom between carboxyl groups; ie, aspartate to glutamate, also changes a strong attractant to a weak attractant. Response of *E. amylovora* to all the attractants were uniformly inhibited by malate by 91–100%. Inhibition of taxis occurs if the attractants share a common receptor site (1). Similarity of chemical structure, all the attractants being three or four carbon dicarboxylic acids, lends further support for the one receptor site hypothesis. Definite conclusions, however, can only be obtained by reciprocal inhibition of all the attractants and finally by the isolation of receptor sites.

Aspartate and other organic acids at 10^{-1}M reduced the motility of *E. amylovora*. Despite this reduced motility, peak responses of all the attractants, except malate, was at 10^{-1}M. In *E. coli*, inhibition of motility due to high ionic environment is accompanied by a corresponding reduction in chemotaxis toward

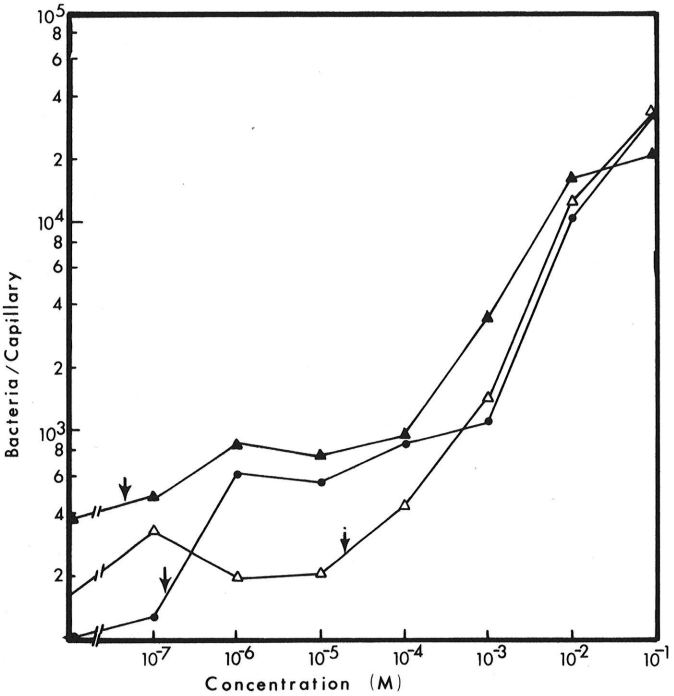

Fig. 3. Concentration response curves for *Erwinia amylovora* attraction to malonate (Δ), succinate (•), and oxaloacetate (▲). Arrows denote response at threshold (accumulation in the absence of attractant plus standard deviation; 67 for malonate, 37 for succinate, and 62 for oxaloacetate) extrapolated for linear portions of the curves. Assays were run for 30 min at 23 C with 8×10^6 cells per milliliter outside the capillary.

that particular compound (2). This peak response at a concentration causing reduced motility may be due to the existence of two chemoreceptor sites, one that senses at lower concentrations and another that senses at higher concentrations as was suggested for *B. subtilis* (14). This would explain why response curves for most of the attractants do not drop at high concentrations. Hence, even if there is reduced motility at 10^{-1}M, if chemoreceptors are not yet saturated, the bacteria will still move toward the attractant but at a slower rate.

The responsiveness of *E. amylovora* to organic acids and aspartate but not to sugars or to the other amino acids appears unique to this organism. Taxis toward organic acids has not been reported in any plant pathogenic bacterium. Such responses are not due to the uniqueness of our strain since the same pattern of taxis was observed with the ATTC strain. The limited range of response of *E. amylovora* may have evolutionary and ecological significance. *Erwinia amylovora* may have had receptor sites for sugars and the other amino acids, but through evolution and adaptation to an ecological niche different from that of the saprophytes, it has lost these receptor sites. Perhaps it has developed unique chemoreceptors for dicarboxylic acids. All the strong attractants, except maleate, are reportedly present in plants and malate, the strongest attractant, accumulates in cell vacuoles of apples (16). Conceivably, this compound or a similar one is the "chemical odor" which *E. amylovora* follows to the portal of entry.

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