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Physiology of Behavioral Mutants of *Rhizobium meliloti*: Evidence for a Dual Chemotaxis Pathway

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Wild-type and nonchemotactic mutant strains of *Rhizobium meliloti* were tested for attraction to localized sites on alfalfa roots and for attraction to numerous small molecules, including sugars, amino acids, and two fractions derived from alfalfa root extracts. Four strains (carrying mutations *che-6*, *che-11*, *che-12*, and *che-26*) lost all responses and were classified as generally nonchemotactic mutants. One strain (carrying mutation *che-7*) lost responses to a group of structurally unrelated amino acids but retained all other responses and was classified as a putative sensory transducer mutant. Two strains (carrying mutations *che-1* and *che-3*) lost responses to all the amino acids and sugars tested but retained normal responses to localized sites on roots and to the root fractions. These two mutant strains could not be classified according to the generally accepted model for a sensory pathway, derived from studies of enteric bacteria, and provided evidence for a dual chemotaxis pathway in *R. meliloti*.

Rhizobia are bacteria which must be adapted to two very different lifestyles. They live in the soil in competition with other soil bacteria, and they live in the root nodule, a private environment where they are supplied by the host plant with useful food (photosynthate) in exchange for the generation of NH_3 from N_2. Since rhizobia typically carry the complicated machinery for motility and chemotaxis, it seems logical that they use the machinery to compete in the soil or to facilitate the infection process which initiates the transition to life in the nodule (4).

Using various species of *Rhizobium*, we and others have described the isolation of behavioral mutants and the use of these mutants to investigate the role of behavior in the infection process (2, 3, 6a, 9, 12, 15). We have also used these mutants to clone the wild-type genes involved (22). In this paper, we present a phenotypic analysis of representative nonchemotactic mutants of *Rhizobium meliloti*. Some unexpected results lead us to suggest a dual chemotaxis pathway with individual branches specialized for the detection of food and the detection of an attractant which guides the bacteria to localized sites on the surface of alfalfa root tips (9).

MATERIALS AND METHODS

Strains. The strains of *R. meliloti* used in this investigation are listed in Table 1. We used wild-type strain Su47 as the standard for all behavioral physiology. Wild-type strain Rm2011, which we formerly used as a standard strain and for the isolation of mutants, is a streptomycin-resistant mutant of Su47, isolated by J. Denarie. We prefer Su47 because it grows somewhat faster and because more of the cells are motile. Some of the mutations originally isolated in Rm2011 were transduced with phage ϕM12 into Su47 by using linkage to Ω26::Tn*5*, an insertion mutation which causes no known phenotypic change other than neomycin and streptomycin resistance (8, 22).

Media and chemicals. The various soft agar swarm media used were based on the minimal swarm medium described previously (3), except that the amount of $(NH_4)_2SO_4$ was reduced 10-fold to 1.5 mM. Synthetic amino acids, obtained from K & K Laboratories, a division of ICN Pharmaceuticals Inc., were used for all behavioral tests so that they would not be confused by contaminants often present in amino acids purified from protein hydrolysates (11).

Traditional swarm plates. The responses of the wild-type strain to sugars and amino acids were originally measured by using soft agar swarm plates in which the attractant is incorporated throughout the test plate and metabolism of the attractant by the bacteria creates the gradient that is detected and ultimately responded to by the bacteria. Positive responses were found for all 20 normal amino acids and numerous sugars (including galactose, glucose, glycerol, lactose, maltose, mannitol, and xylose). For each compound tested, a concentration was determined which gave a maximal response under conditions with that compound as the sole carbon source. In some cases, the results were unclear because the bacteria grew poorly at the concentrations which gave observable attraction. The appropriate solution to this problem is the incorporation into the media of a carbon source which supports good growth but is not itself an attractant. For example, glycerol is used routinely with *Escherichia coli*. Unfortunately, we have not found a carbon source which can be used in this manner; all of the candidate compounds tested which supported good growth were also attractants. Instead, we incorporated high levels of maltose (0.4%) into the swarm plates to support growth. As expected, at these high levels the response to maltose was greatly reduced, presumably because the system for responding to maltose was saturated. In response to amino acids present in low concentrations, the bacteria formed a diffuse ring outside a central area of heavy growth. Although not ideal, this system could be used for the preliminary attractant survey and the enrichment of mutants.

Point source swarm plate assay. Numerous assays, each with advantages and disadvantages, can be used for the measurement of chemotaxis in bacteria (1, 5, 19). To survey the responses of wild-type and mutant strains of *R. meliloti* to numerous potential attractants and to test fractions from plant root extracts, we required an assay that was rapid, quantitative, and suitable for use with nonmetabolizable

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TABLE 1. *Rhizobium* strains used

Strain	Genotype (background strain)	Isolation method	Source (reference)
Su47	Wild type		
Rm2011	Wild type		
NR8000	Ω26::Tn*5* (Su47)		C. Peirce (22)
NR3000	*che-1* (Rm2011)	Spontaneous	P. Ames (3)
NR8003	*che-1* Ω26::Tn*5* (NR3000)	NR8000 × NR3000[a]	
NR8006	*che-1* Ω26::Tn*5* (Su47)	NR8003 × Su47	
NR4000	*che-3* (Rm2011)	Spontaneous	R. LaRosiliere
NR8004	*che-3* Ω26::Tn*5* (NR4000)	NR8000 × NR4000	
NR8007	*che-3* Ω26::Tn*5* (Su47)	NR8004 × Su47	
NR4300	*che-6* (Rm2011)	Spontaneous	R. LaRosiliere
NR4400	*che-7* (Rm2011)	Spontaneous	R. LaRosiliere
NR3111	*che-11* (Su47)	Tn*5* mutagenesis	P. Ronco
NR3112	*che-12* (Su47)	Tn*5* mutagenesis	P. Ronco
NR3126	*che-26* (Su47)	Tn*5* mutagenesis	P. Ronco

[a] Transductional crosses with phage φM12 are listed with the donor strain first.

attractants. After numerous preliminary experiments, in some cases complicated by technical difficulties peculiar to our strain of *Rhizobium*, we eventually settled on a modified swarm plate assay. Traditional swarm plate assays, in which metabolism of the attractant by the bacteria creates the gradient, are of limited usefulness for quantitative measurements of responses to metabolizable attractants and are useless for the measurement of responses to nonmetabolizable attractants. We solved these problems by incorporating the substance to be tested into a controlled-release polymer vehicle (13) or by drying the substance onto filter paper disks. Bacteria from a single colony of the wild type or mutant strain to be tested were stabbed into a soft agar plate containing high levels of maltose or glucose (0.4%) as the carbon source. After overnight growth, disks containing the sample to be tested were placed on the plate at 1.5 cm from the point of inoculation. Between 24 and 48 h later, the movement of bacteria toward the disk, indicated by a flare in the direction of the disk, could be scored. Figure 1 shows the response of the wild type and mutants to filter paper disks containing a crude fraction from a root tip extract (panel a) and serine (panel b).

Mutant isolation. The Tn*5* insertions listed were derived from conjugations between Su47 and *E. coli* MM294A (pRK602), provided by T. Finan. pRK602 is a Tn*5* delivery vector derived from pRK2013 (14). Transconjugants with Tn*5* insertions were selected on LB medium containing 250 μg of streptomycin and 200 μg of neomycin per ml. A mixed culture derived from at least 5,000 transconjugant colonies was enriched for behavioral mutants by allowing the culture to swim out on yeast extract swarm plates and by picking cells which had moved only slightly (approximately 2 mm) from the inoculation site (3). After only one round of enrichment, single colonies were tested for behavioral phenotype. Nonmotile mutants were isolated from 1 of 40 colonies screened. Nonchemotactic mutants were isolated

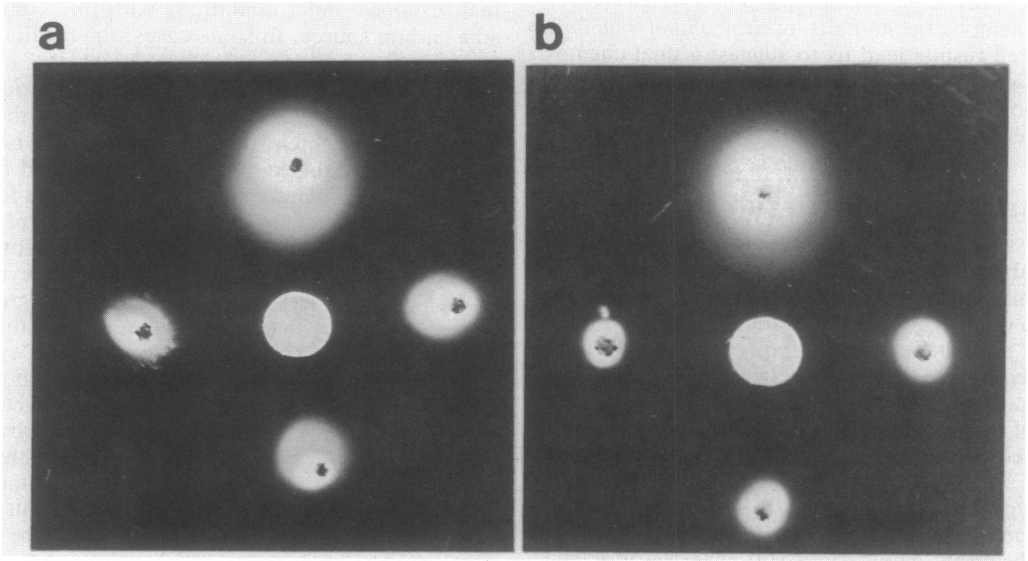

FIG. 1. Photographs of point source swarm plate assays. In both pictures, the strains are (clockwise from the top) Su47 and mutants carrying *che-3*, *che-1*, and *che-11*. The filter paper disks received 8 μl of a 1/50 dilution of root extract fraction A2 (a) and 0.1 mM serine (b). So that the response would be evident in the photographs, charcoal was added with the bacteria to mark the inoculation sites. The photographs were taken 48 h after the filter paper disks were placed in position. A positive response was scored for all strains except the strain carrying *che-11* in panel a and for only Su47 in panel b.

from 1 of 250 colonies screened. All of the other mutants were isolated without mutagenesis following multiple rounds of enrichment on yeast extract swarm plates or minimal swarm plates containing histidine or serine as the attractant (3).

Repeatable tests for attraction of rhizobia to localized sites on alfalfa roots. We have previously reported our observations, documented with a video recording system, of rhizobial behavior in the vicinity of plant roots (9). The plant roots are placed in slide chambers and inoculated with a motile culture of rhizobia. Within 15 min, bacteria can be observed to accumulate at highly localized sites on the surface of the root. The bacteria in the accumulation are motile and appear to be swimming toward a point source of attractant. The accumulations are small and roughly hemispherical, with a radius of 50 to 200 μm, which corresponds to a volume of 0.2 to 8 nl.

To test the effects of bacterial mutations and added substances (including root exudates, fractions derived from root extracts, and known compounds) on this phenomenon, we must be able to measure it repeatedly. However, the number of accumulations per root is low and highly variable. The solution to this problem depended on the observation that following rinsing of the plant with motility buffer, newly introduced bacteria accumulated at exactly the same site. The washing and rechallenging could be performed repeatedly.

Thus, we set up a strict experimental protocol to test the effects of changes in conditions or bacterial strain as follows: challenge 1, Su47, standard conditions, observe accumulation; challenge 2, change in strain or conditions, observe result; challenge 3, Su47, standard conditions, observe accumulation. By following this protocol, we had a well-controlled test of the variable in challenge 2, whether the result was positive or negative.

Fractionation of root extract. Strong cation-exchange resin AG 50W-X4 was obtained from Bio-Rad Laboratories. High-performance liquid chromatography was performed on a Varian Model 5020 liquid chromatograph. The reverse-phase column (C18; Supelco) was used with sodium dodecyl sulfate as a strong ion pair (10). The weak anion-exchange column (Micro-Pak AX5; Varian) was used with the volatile buffer system described by Dizdaroglu (7). The most efficient fractionation we used was an isocratic run in 40% 0.01 M triethylammonium acetate (pH 5.0)–60% acetonitrile.

RESULTS

Survey of the responses to amino acids of wild-type and mutant strains. Table 2 lists the amino acid attractants tested in the filter paper assay. The potencies of the attractants for the wild type and strains carrying mutations were compared by determining the minimal concentration of attractant which gave a detectable response in the filter paper assay. Strains carrying the generally nonchemotactic mutations *che-6*, *che-11*, *che-12*, and *che-26* were completely nonresponsive (data not shown). As shown in the table, strains carrying *che-1* and *che-3* were nonresponsive except to higher concentrations of methionine and histidine and, in the case of the strain carrying *che-1*, glutamine and threonine. The strain carrying *che-7* responded normally to some amino acids (aspartate, histidine, isoleucine, proline, serine, threonine, tyrosine, and valine) and not at all to others (alanine, arginine, glutamate, glutamine, phenylalanine, and tryptophan). It responded with an altered threshold to asparagine, cysteine, cystine, glycine, leucine, and methionine.

TABLE 2. Minimum amino acid concentration on filter paper needed to attract strain

Amino acid	Minimum concn (mM) needed to attract the following strain:			
	Wild type	Mutant carrying:		
		che-1	*che-3*	*che-7*
Alanine	1	>10	>10	>10
Arginine	0.01	>10	>10	>10
Asparagine	10	>100	>100	100
Aspartate	0.01	>10	>10	0.01
Cysteine	0.1	>10	>10	1
Cystine	0.1	>10	>10	1
Glutamate	0.1	>10	>10	>10
Glutamine	0.001	100	>100	>10
Glycine	1	>100	>100	10
Histidine	0.01	10	10	0.01
Isoleucine	0.1	>100	>100	0.1
Leucine	1	>100	>100	10
Lysine	0.1	>10	>10	>10
Methionine	0.1	1	1	1
Phenylalanine	1	>100	>100	>100
Proline	1	>10	>10	1
Serine	0.1	>100	>100	0.1
Threonine	0.01	10	>10	0.01
Tryptophan	0.1	>10	>10	>10
Tyrosine	0.1	>10	>10	0.1
Valine	1	>100	>100	1

Effect of behavioral mutations on the accumulation at localized root sites. We observed the behavior of each of the strains in the vicinity of alfalfa roots by using the strict protocol detailed above. The strain carrying *che-7*, which has lost responses to a subset of the normal amino acid attractants, was still attracted to root sites. The strains carrying *che-6*, *che-11*, *che-12*, and *che-26*, which have lost responses to all attractants tested, were not attracted to root sites. Our expectation was that any other *che* mutants which failed to respond to all known compounds tested would be similarly defective. However, strains carrying *che-1* and *che-3*, which do not respond normally to any of the known attractants tested (including both amino acids and sugars), were still attracted to the root sites.

Substances that inhibit the accumulation at localized sites. We tested the effect of various potentially competitive attractants on the accumulation at localized sites. Root exudate inhibits the response, presumably because the bacteria can no longer detect an attractant gradient in the presence of a uniform high background. Sugars have no effect even at 0.2%. Individual amino acids inhibit the response at various levels. Table 3 shows the minimum amino acid concentrations needed to inhibit the response in the wild type (Su47) and in mutant strains.

Attractants from plant root tip extracts. Our physiological work has now provided us with two helpful tools to identify the attractant(s) that mediates the response to localized sites on the plant root; (i) the correct substance should block the accumulation of rhizobia at localized sites on alfalfa roots, and (ii) strains carrying *che-1* and *che-3*, which are not attracted to any of the individual small molecules we have tested but still accumulate at the localized sites, can be used instead of the wild type in the filter paper swarm plate assays.

However, it is important to remember that extremely small quantities of an active attractant could be involved, since the volume of the clouds is no more than 10 nl. Our

TABLE 3. Minimum amino acid concentration needed to block accumulation of strain at localized sites on alfalfa roots

Amino acid	Minimum concn (mM) needed to block the following strain:			
	Wild type	Mutant carrying:		
		che-1	*che-3*	*che-7*
Alanine	0.1	0.01	0.1	
Arginine	0.01	0.01	0.01	0.01
Asparagine	5			
Aspartate	>5			
Cysteine	0.001	0.01	0.01	0.001
Cystine	0.01	0.01	0.01	
Glutamate	5			
Glutamine	>5			
Glycine	>5			
Histidine	>5			
Isoleucine	5			
Leucine	>5			
Lysine	0.01	0.001	0.01	0.1
Methionine	>5			
Phenylalanine	1	1	0.1	
Proline	0.1	0.1	0.1	
Serine	>5			
Threonine	0.01	0.01	0.1	0.1
Tryptophan	>5			
Tyrosine	>5			
Valine	0.5			

initial approach was to collect root exudate in 10-μl drops placed over the active region of the plant root. Despite the enormous dilution, material collected and pooled from many individual plants did block accumulation at localized sites. The assay was made quantitative by dilution to an endpoint. The small amount of material collected in this way was sufficient to characterize the active molecule(s) as heat stable in the autoclave, dialyzable, and insoluble in chloroform.

For purification of attractants from roots, we increased the amount of starting material by purchasing 5-lb (ca. 2.3-kg) trays of uniform, highly packed immature (3-day) alfalfa sprouts from a commercial grower. Root exudate was prepared by soaking the root tips in water. Root extract was prepared by grinding the root tips in buffer or water. In both cases, the material was concentrated by lyophilization.

Most of our fractionations have used root extract material because it had a much higher total activity and a slightly higher specific activity. Dilutions of samples were tested in the filter paper assay. One unit of activity was defined as the amount of active material which produced a threshold response in the standard assay (8-μl sample applied to filter paper). The A_{220} was used to estimate the total material present.

Preliminary clean-up steps which typically worked at greater than 95% yield included precipitation of contaminating material with 65% acetone and adsorption onto a strong cation-exchange column (AG50W) in 0.01 M acetic acid, followed by elution with 0.1 M triethylamine. The material was further purified by high-pressure liquid chromatography on a reverse-phase column with sodium dodecyl sulfate as a strong ion pair or on a weak anion-exchange column (Micro-Pak AX5). On each column, the activity was separated into two fractions, called A1 and A2. More extensive work was done with the anion-exchange column because the fractionation was more rapid (15 min) and all of the buffers were volatile. By these methods, fraction A2 has been purified approximately 1,000-fold relative to contaminating material that absorbs at 220 nm. The activities of both fractions were eliminated by derivatization with dansyl chloride.

TABLE 4. Summary of behavioral phenotypes

Behavioral genotype	Revertants isolated?	Swimming pattern[a]	Attraction to:			
			Sugars	Amino acids	Localized sites	Root extract fractions
Wild type		R	+	+	+	+
fla-1	Yes	Nonmotile	−	−	−	−
mot-1	Yes	Nonmotile	−	−	−	−
che-1	Yes	T	−	−	+	+
che-3	Yes	R	−	−	+	+
che-6	Yes	S	−	−	−	−
che-7	Yes	R	+	Some	+	+
che-11		T	−	−	−	−
che-12		T	−	−	−	−
che-26		S	−	−	−	−

[a] The wild-type swimming pattern is a random (R) alternation between smooth swimming (S) and tumbling (T).

The partial purification of attractants from alfalfa root tip extract allowed us to test the sensitivity of wild-type and mutant strains to these materials. In the filter paper assay, strains carrying *che-1*, *che-3*, or *che-7* had exactly the same relative sensitivity as the wild type. Strains carrying *che-6*, *che-11*, *che-12*, or *che-26* were completely nonresponsive.

Summary of physiological and genetic data on mutant strains. In Table 4, we present a summary of the data collected for behavioral mutant strains in this and previously published reports (2, 3, 9). With the exception of strains carrying *che-6* and *che-26*, all of these mutant strains are recessive in diploids, since they were complemented by cloned DNA fragments from a genomic library of *R. meliloti*. Despite repeated efforts, we have not complemented the *che-6* mutation. Since the mutation maps to one side of the cloned *fla-che* region, we do not yet know whether it is dominant or whether the appropriate DNA fragment is not present in the genomic library screened (22).

DISCUSSION

Our analysis of motility and chemotaxis in *R. meliloti* has been strongly influenced by the model for a sensory pathway developed for the enteric bacteria. An important element of the generally accepted model is peripheral integration of sensory inputs to a common output system. Information from numerous receptors is integrated by a few sensory transducer proteins, communicated through a common *che* system, to control the direction of rotation of the flagellum motors (6, 17, 18).

In agreement with these models, we have isolated and characterized mutants which are examples of the expected classes: nonmotile mutants (carrying both *fla* and *mot*) with defective flagellum motors, generally nonchemotactic mutants, including smooth swimmers (carrying *che-6* and *che-26*) and tumbly swimmers (carrying *che-11* and *che-12*) with defective common *che* systems, and a putative signal transducer mutant (carrying *che-7*), which has lost responses to a subset of structurally unrelated amino acids that are probably detected by different chemosensors.

However, the behavior of mutant strains carrying *che-1* and *che-3* does not seem to fit. How can these strains have lost responses to all the amino acid and sugar attractants tested, so that they appear to be generally nonchemotactic,

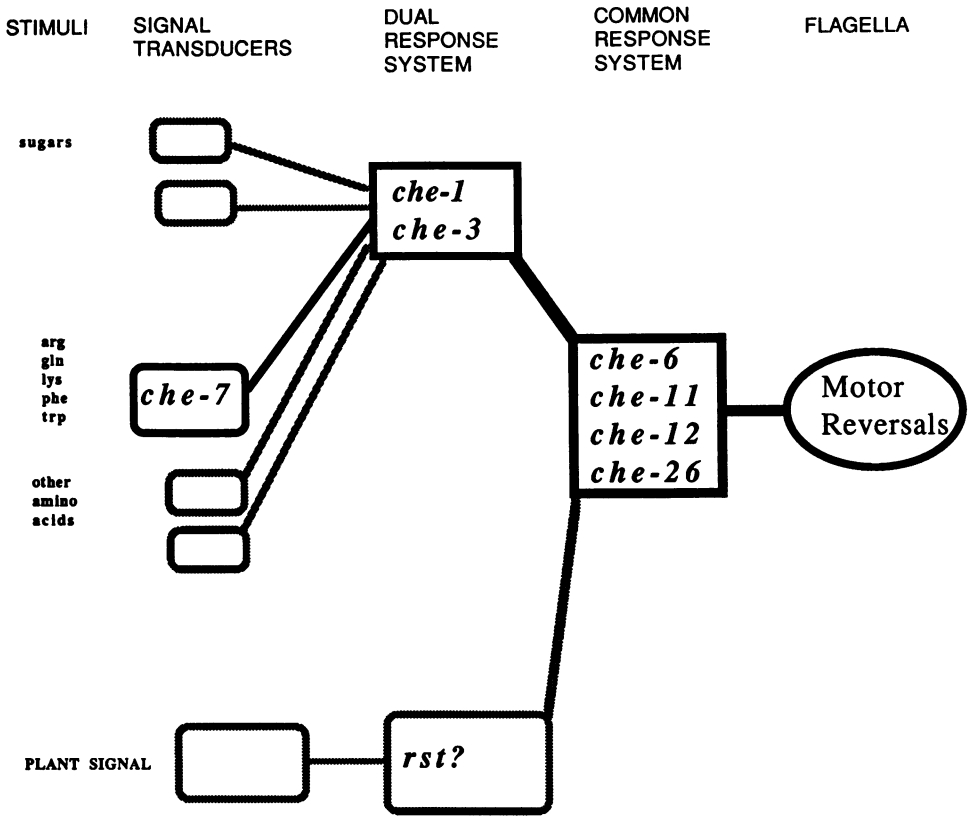

FIG. 2. Dual chemotaxis pathway model. Predicted components of the pathway for which mutants have not yet been isolated are shown in gray.

and yet have retained the qualitative response to localized sites on the plant root and the quantitative response to the A1 and A2 fractions isolated from plant roots? Although the precise molecular nature of the *che-1* and *che-3* mutations is unknown, we do know that both mutations are spontaneous, are recessive, and revert. Furthermore, the phenotypes are caused by a single mutation, since strains isolated as revertants for one of the responses (for example, attraction to maltose or histidine) have reverted simultaneously for all the other responses (R. C. LaRosiliere, M.S. thesis, Northeastern University, Boston, Mass., 1984).

A conceptual framework which we have found useful to think about these results is the dual pathway model diagrammed in Fig. 2. One branch of the dual pathway would be specialized for the detection of food, and the other branch would be specialized for detection of a signal, the localized site attractant, involved in establishing symbiosis with the plant. The mutations *che-1* and *che-3* would specifically affect the food detection pathway. Although these mutations cause recognizably different phenotypes (cells carrying *che-1* tumble continuously except near the plant, while cells carrying *che-3* have normal random tumbling and smooth swimming), they are closely linked and may even be different changes in one gene (22). The generally nonchemotactic mutants (carrying *che-6*, *che-11*, *che-12*, and *che-26*) would affect the elements which are shared by both branches of the dual pathway. Mutations in the root site taxis pathway (putative *rst* mutants) remain to be isolated.

The blocking of the accumulation at localized sites by amino acids must also be explained. Since the blocking potency of individual amino acids is the same (except for some small quantitative differences) for the wild type and strains carrying *che-1*, *che-3*, and *che-7* which lack elements of the food pathway, we propose that blocking is mediated by the root site taxis pathway, presumably by competition at the chemosensor or transducer for the true localized site attractant.

The dual pathway hypothesis does not require that both chemotaxis pathways be expressed in each cell of a strain; in fact, only a small minority of the cells near the plant accumulate in the localized sites. Specialization in cells of a single culture is now well known. Some relevant examples are the flagellar system of *Salmonella typhimurium* (21) and the plant attachment system of *Bradyrhizobium japonicum* (20).

There is clearly a danger of overinterpreting what is still a fragmentary picture developed from the physiological analysis of a limited number of behavioral mutants and from the analogy with enteric bacteria. Some less likely but formally acceptable possibilities are the following.

(i) All of the amino acid and sugar attractants may be processed through the same signal transducer that is defective in *che-1* and *che-3*. Two observations tend to rule this out; chemotaxis to amino acids is unaffected by high levels of maltose, and the strain carrying *che-7* already has the characteristics of a signal transducer mutant but affects responses to a smaller subset of attractants.

(ii) The *che-1* and *che-3* mutations may be leaky point mutations in a central response gene and may produce enough gene product to maintain chemotaxis to attractants from the plant but not to sugars or amino acids. This is unlikely since we have now isolated a Tn*5* insertion muta-

tion, presumably a null mutation, which causes exactly the same phenotype as that caused by *che-1* (E. Nulty, unpublished data).

(iii) The *che-1* and *che-3* mutations may be in a gene which controls the whole set of genes that make sensory transducer proteins involved in the response to amino acids and sugars. Since *che-1* and *che-3* are distant from *che-7* (22) this could not be due to polar effects on transcription.

(iv) The *che-1* and *che-3* mutations may affect an adaptation system, presumably involving transducer methylation, which is not used in the root site taxis pathway. In fact, there is evidence for alternate, methylation-independent, adaptation pathways in the response of enteric bacteria to oxygen and sugars transported by the phosphotransferase system (16).

The true explanation of these results will require the isolation and analysis of a more complete set of behavioral mutants, including definitive null mutations in the *che-1* or *che-3* gene (or both genes).

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