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Relationships between C_4 Dicarboxylic Acid Transport and Chemotaxis in *Rhizobium meliloti*

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The relationship between chemotaxis and transport of C_4 dicarboxylic acids was analyzed with *Rhizobium meliloti dct* mutants defective in one or all of the genes required for dicarboxylic acid transport. Succinate, malate, and fumarate were moderately potent chemoattractants for wild-type *R. meliloti* and appeared to share a common chemoreceptor. While dicarboxylate transport is inducible, taxis to succinate was shown to be constitutive. Mutations in the *dctA* and *dctB* genes both resulted in the reduction, but not elimination, of chemotactic responses to succinate, indicating that transport via DctA or chemosensing via DctB is not essential for C_4 dicarboxylate taxis, although they appear to contribute to it. Mutations in *dctD* and *rpoN* genes did not affect taxis to succinate. Aspartate, which is also transported by the dicarboxylate transport system, elicited strong chemotactic responses via a chemoreceptor distinct from the succinate-malate-fumarate receptor. Taxis to aspartate was unaltered in *dctA* and *dctB* mutants but was considerably reduced in both *dctD* and *rpoN* mutants, indicating that aspartate taxis is strongly dependent on elements responsible for transcriptional activation of *dctA*. Methylation and methanol release experiments failed to show a significant increase in methyl esterification of *R. meliloti* proteins in response to any of the attractants tested.

Because both chemotaxis and active transport systems contribute to nutrient acquisition by bacteria, it is of interest to determine whether taxis and uptake activities are coordinated in some manner or share components in their signal transduction pathways. Evidence of coordinated chemotaxis and uptake has been reported previously. Ingham and Armitage (19) suggested that transport of attractants was required for chemotactic sensing in *Rhodobacter sphaeroides*. In *Pseudomonas putida*, Karimian and Ornston (20) showed that both the chemotactic response and transport systems for β-ketoadipate were inducible. Regulatory mutants that synthesized the transport system at high levels exhibited enhanced chemotaxis to β-ketoadipate. The regulation of chemotactic responsiveness can be quite complex and often indirect, as demonstrated by the induction of benzoate chemotaxis by β-ketoadipate in *P. putida*, a metabolite seven enzymatic steps removed from the parent molecule (18).

For the present paper, we examined the possibility of coordination between chemotaxis to and uptake of dicarboxylic acids and aspartate in *Rhizobium meliloti*. The chemotactic responses of *R. meliloti* to various dicarboxylic acids were measured and then compared with the responses of mutants defective in one or all of the genes required for dicarboxylic acid transport. A number of bacteria, including *Escherichia coli*, *Salmonella* species, *R. sphaeroides*, *Rhizobium leguminosarum* and *Bradyrhizobium japonicum* are chemotactically responsive to carboxylic acids (2, 14, 19, 25, 26, 30). Chemotaxis to the C_4 dicarboxylic acids is of special interest in *Rhizobium* species because the C_4 dicarboxylic acids play important roles as substrates and signal compounds for these symbiotic, nitrogen-fixing bacteria. C_4 dicarboxylic acids such as succinate, malate, and fumarate are considered to be the major carbon sources utilized by both free-living rhizobia and differentiated bacteroids in root nodules and support the fastest growth rates in laboratory culture (7, 10, 12, 24, 44). C_4 dicarboxylic acids may also play a key role in initiating the differentiation of free-living cells to bacteroids in nodules of alfalfa and red clover (13, 45).

In *Rhizobium* species, succinate, malate and fumarate are transported by a common C_4 dicarboxylate transport (Dct) system (11, 23, 34, 39, 46). Transport is carrier mediated, active, and generally inducible. Aspartate also appears to be transported via the Dct system in *R. meliloti* (46). In *R. meliloti* and *R. leguminosarum*, three genes are associated with dicarboxylate transport, *dctA*, *dctB*, and *dctD* (34, 35). *dctA* encodes the transport protein, DctA (6, 34). The *dctB* and *dctD* gene products are regulatory proteins which appear to be constitutively synthesized at low levels. It has been proposed that the DctB protein spans the cytoplasmic membrane and is activated by binding to C_4 dicarboxylic acids. The active DctB then turns on the *dctD* gene product DctD, which in conjunction with the alternative sigma factor *rpoN* activates transcription of the transport protein DctA (34, 36). DctB and DctD are members of a conserved family of two-component regulatory systems, which includes the chemotaxis proteins CheA, CheY, and CheB (37, 43).

MATERIALS AND METHODS

Bacterial strains, media, and buffers. Wild-type *R. meliloti* JJ1c10 (Rif^r Dct$^+$) and the dicarboxylate transport mutants 4F6 (*dctA*::Tn*5* Rif^r Km^r Dct$^-$) and R639 (*dctB*:Tn*5* Rif^r Km^r Dct$^-$) were provided by Robert J. Watson (Plant Research Center, Agriculture Canada, Ottawa, Canada) and have been previously described (46). *R. meliloti* 1021 (wild type) and the *dct* deletion mutant of this strain, *R. meliloti* F726, were obtained from T. Finan (Department of Biology, McMaster University, Hamilton, Ontario, Canada) and have been previously described (10). *E. coli* chemotaxis wild-type strain RP437 and the Tar deletion mutant RP2361 were obtained from J. Parkinson (Department of Biosciences, University of Utah), as previously described (31). Stocks of all *R. meliloti* cultures were maintained as stab cultures in

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TY (tryptone-yeast extract) soft agar at 4°C and subcultured every 6 to 8 weeks. Long-term storage consisted of glycerol stocks stored at −80°C. *E. coli* strains were cultured on tryptone agar and maintained as glycerol stocks stored at −80°C.

R. meliloti was grown in NM minimal salts medium (9) supplemented with 0.2 ml of a trace metal solution and 1.0 ml of Gotz (15) vitamin solution. Mannitol was added as the carbon source at 20 mM except where noted. The medium was adjusted to pH 7.0 prior to autoclaving. Both the $CaCl_2$ and vitamins were added to the medium after autoclaving. The trace metal solution, clarified with HCl, consisted of (per liter) 1.4 g of EDTA, 2.2 g of $ZnSO_4 \cdot 7H_2O$, 0.39 g of $MnSO_4 \cdot H_2O$, 0.08 g of $CuSO_4 \cdot 5H_2O$, 1.3 g of $Na_2MoO_4 \cdot 2H_2$), 0.12 g of $CoCl_2 \cdot H_2O$, 0.22 g of $Na_2B_4O_7 \cdot OH_2O$, 0.465 g of Na_3VO_4, and 0.04 g of Na_2SeO_3. TY medium used for stock cultures and plate counting contained (per liter) 6.0 g of tryptone, 3.0 g of yeast extract, and 0.5 g of $CaCl_2$. Shake-flask cultures were grown at 30°C in a rotary shaker at 175 rpm. Chemotaxis buffer consisted of 10 mM potassium phosphate or HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid) (pH 7.0), 10 μM EDTA (disodium salt), and 100 μM $CaCl_2$ in high-performance liquid chromatography-grade water (33). The $CaCl_2$ was added as a sterile solution after autoclaving.

Chemotaxis assays. Motile, logarithmic-phase *R. meliloti* cells were harvested at an A_{590} of 0.15 to 0.20. Cells were centrifuged at 1,900 × *g* for 15 min and washed once with chemotaxis buffer. Washed cells were gently resuspended in chemotaxis buffer to a density of ca. 1.0×10^8 cells per ml. Chemotaxis buffer was shaken vigorously prior to use to ensure aeration. Motility was assessed microscopically with a Petroff-Hausser bacterium-counting chamber by counting the number of motile cells in the plane of the calibrated grid. Generally, 80 to 90% of the washed cells were motile and remained motile for at least 2 h.

Chemotaxis was measured with capillary assays (1, 4) in the chemotaxis chambers described by Palleroni (29). Measurements were made by placing 1-μl capillaries (Microcaps; Drummond Scientific, Broomall, Pa.) filled with chemotaxis buffer or buffer plus attractant into chambers filled with bacteria resuspended in chemotaxis buffer as described above and then incubating them without vibration at 28°C for 60 min. At the end of this period, the capillary tubes were removed with forceps and rinsed gently with sterile distilled water, and their contents were diluted into phosphate-buffered saline and plated onto TY agar plates with a model DU plating instrument (Spiral Systems, Inc., Cincinnati, Ohio). Plates were incubated at 30°C for 3 days. All chemotaxis assays were performed in triplicate or quadruplicate and repeated at least once. The number of bacteria entering control, buffer-filled capillaries ranged from 2,800 to 6,100. The definitions of Mesibov and Adler (26) for threshold concentration, peak response, peak concentration, and concentration-response curve are used here. Threshold values were calculated from log-log plots of bacteria accumulated versus concentration for a range of concentrations of attractant. A straight line was fitted by linear regression to the points on the rising portion of the concentration-response curves. The concentration at which that line intersected the baseline, defined by the background accumulation in capillaries containing no attractant), was taken as the threshold value. Chemotaxis competition experiments, in which the ability of one compound to block or inhibit chemotaxis of the bacteria to another compound was measured, were performed by adding the potential inhibitor or competitor to both the capillary tube and the well.

In vivo methylation. Protein methylation assays were based on published procedures (2, 17, 22). Cultures of motile cells in the logarithmic phase of growth in NM minimal salts medium were harvested, washed once, and suspended in chemotaxis buffer (10 mM HEPES [pH 7.0], 10 μM disodium EDTA, 0.2 mM $CaCl_2 \cdot 7H_2O$), to a concentration of 10^8 cells per ml. After being incubated for 15 min at 28°C in the presence of 200 μg of chloramphenicol per ml, 1.0-ml samples of the cell suspension were aliquoted into microcentrifuge tubes, and 10 μCi of L-[*methyl*-^{3}H]methionine was added (specific activity, 75 Ci/mmol). After 60 min, 5-μl additions of buffer or attractants were made (final concentrations of attractants varied between 5 and 20 mM). After an additional brief incubation period, cells were harvested in a microcentrifuge, and methionine incorporation was arrested by resuspending the cell pellets in sodium dodecyl sulfate (SDS) sample buffer and boiling them for 5 min. Samples of solubilized cells were separated by SDS-polyacrylamide gel electrophoresis (PAGE) (0.75-mm gels, 10% resolving gel). Gels were stained with Coomassie blue, soaked in En^{3}Hance, dried for 3 h under vacuum at 60°C, and exposed to X-ray film at −80°C for 5 to 15 days.

Methanol release. The assay described by Chelsky and Dahlguist (5) was used to determine whether there are methyl-esterified proteins in *R. meliloti* similar to the methyl-accepting chemotaxis proteins (MCPs) of *E. coli*. Cells were cultured and processed and the proteins were separated by SDS-PAGE just as described for the in vivo methylation experiments. After staining with Coomassie blue and destaining as described above, each lane was sliced with a razor blade into 2 mM slices in the 43- to 97-kDa range. Each gel slice was transferred to a capless 1.5-ml microcentrifuge tube, and then the tube was set inside a 10-ml scintillation vial containing 5 ml of scintillation cocktail. Sodium hydroxide (0.2 ml of an 8 N solution) was added to the Microfuge tubes containing the gel slices, and the scintillation vial was immediately stoppered. The vials were incubated at room temperature (24°C) for 24 h to allow the [^{3}H]methanol released to equilibrate with the scintillation fluid before counting.

Chemicals. The following chemicals were tested as chemoattractants: succinate (disodium salt), malate (disodium salt), fumarate (disodium salt), itaconic acid (disodium salt), aspartic acid, L-glutamine, and cycloleucine. These chemicals were purchased from ICN Pharmaceuticals, Inc. L-[*methyl*-^{3}H]methionine was obtained from Amersham Corp. The electrophoresis chemicals acrylamide, methylene bisacrylamide, SDS, glycine, *N,N,N′,N′*-tetramethylethylenediamine, and ammonium persulfate were all of electrophoresis purity (Bio-Rad Laboratories). Protein molecular weight standards were purchased from BRL, Inc.

RESULTS

Chemotaxis of *R. meliloti* to C_4 dicarboxylic acids. The chemotactic responses of *R. meliloti* JJ1c10 grown on NM mannitol medium to aspartate, succinate, malate, fumarate, and itaconic acid are shown in Table 1 and Fig. 1. Itaconic acid (3-carboxy-3-butanoic acid) is a nonmetabolizable analog of succinate (32). Chemotactic responses to cycloleucine were tested because it is a compound known to cause inhibition of chemotaxis in *E. coli*, and glutamine was included as a positive control in all experiments.

R. meliloti JJ1c10 showed maximum responses to fuma-

TABLE 1. Chemotactic responses of *R. meliloti* JJ1c10 to dicarboxylic acids and amino acids

Attractant	Concn of attractant (mM)	Maximal chemotactic response (mean no. of cells/capillary ± SD)[a]	Threshold (M)[b]
Succinate	75	39,000 ± 6,500	2.6×10^{-4}
Malate	75	41,000 ± 2,300	7.9×10^{-4}
Fumarate	100	51,000 ± 2,600	1.7×10^{-3}
Itaconic acid	100	70,000 ± 13,000	7.8×10^{-4}
Aspartate	100	120,000 ± 7,600	2.5×10^{-5}
Glutamine	50	394,000 ± 40,000	8.3×10^{-6}
Cycloleucine	100	1,200,000 ± 73,000	2.0×10^{-7}

[a] Values are averages from two separate trials, each performed in quadruplicate. Background accumulations varied from 2,800 to 6,100 bacteria per capillary.

[b] Threshold concentrations were calculated from log-log plots of bacteria per capillary versus the concentration of attractant (26).

rate, itaconic acid, aspartate, and cycloleucine at 100 mM, the highest concentration tested (Table 1), while responses to succinate and malate peaked at 75 mM. The peak response to glutamine was shown to be at 50 mM. Responses to the dicarboxylic acids ranged from 6- to 18-fold over background, making them moderately potent attractants, weaker than the amino acids aspartate, glutamine, and cycloleucine, but stronger attractants than glucose or the *nod* gene inducing plant flavonoids (2, 4, 25). Threshold concentrations, defined as the lowest concentration of attractant that gives an accumulation in the capillary greater than the background level (26), were similar for the different dicarboxylic acids, ranging from about 2×10^{-3} to about 3×10^{-4} M.

Growth of strain JJ1c10 on NM medium containing 20 mM succinate did not significantly enhance its chemotactic responsiveness to succinate. Similarly, 1 h of prior exposure to 20 mM succinate or aspartate, levels sufficient to induce the *dct* transport system (46), had no measurable effect on chemotaxis to succinate or aspartate, indicating that dicarboxylate chemotaxis, unlike transport, is not inducible in this organism.

Unexpectedly, cycloleucine was found to be a very potent chemoattractant for *R. meliloti* JJ1c10. However, even with chemotaxis ratios in excess of 100, peak responses were recorded at 100 mM, the highest concentration tested. These results indicate that, as with other attractants tested, saturation had not been reached. Cycloleucine had the lowest threshold concentration of the compounds tested. A threshold of 2.0×10^{-7} M for cycloleucine is 40-fold lower than the threshold for glutamine, the next most potent attractant.

Chemotaxis competition experiments. To determine whether *R. meliloti* has a single chemoreceptor for the dicarboxylic acids or has two or more independent receptors, competition assays were performed. Malate, aspartate, glutamine, or cycloleucine (10 mM each) was added to both the capillary and the well to determine its effect on various attractants present in the capillaries at an equal or greater concentration. In these experiments, the general motility of JJ1c10 cells was reduced by 5 to 10% by the addition of succinate, malate, or fumarate. In contrast, aspartate, glutamine, and cycloleucine did not have an inhibitory effect on general motility. The basis for this selective effect on motility is not known but was minimized in competition experiments by adding the motility-reducing competitors at 10 mM, which is well below the peak concentrations noted in Table 1 and is below the concentrations of the attractants in the capillaries.

As shown in Table 2, malate inhibited chemotaxis to all compounds tested. However, taxis to succinate was strongly inhibited compared with that to the other compounds. At a concentration of 10 mM, malate was able to reduce responses to 50 mM succinate by 10-fold. Malate also appears to have had a rather general effect on responsiveness to various attractants.

In similar experiments, 10 mM aspartate was found to inhibit taxis toward aspartate, glutamine, and cycloleucine but did not inhibit taxis to succinate or malate (Table 2). Response ratios of <1 represent increased accumulations in

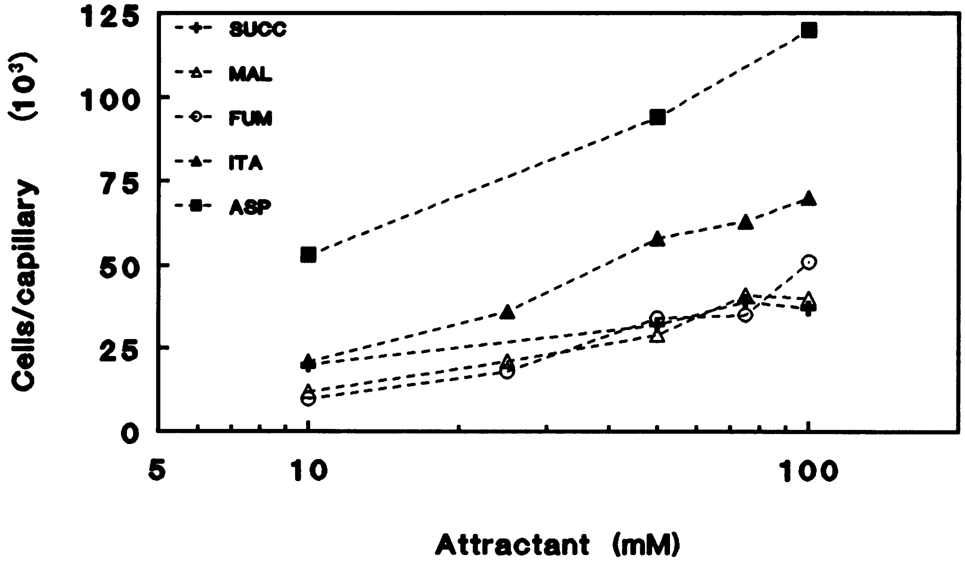

FIG. 1. Concentration-response curves for chemotaxis of *R. meliloti* JJ1c10 to succinate (SUCC), malate (MAL), fumarate (FUM), itaconic acid (ITA), and aspartate (ASP). Results are the averages of two separate trials performed in quadruplicate.

TABLE 2. Inhibition of chemotaxis in *R. meliloti* JJ1c10

Competitor (10 mM)	Attractant[a]	Mean no. of cells/capillary ± SD[b]		Response ratio[c]
		Buffer	Buffer + competitor	
Malate	Malate	58,000 ± 13,000	8,400 ± 1,000	6.9
	Succinate	59,000 ± 17,000	5,900 ± 900	10
	Fumarate	42,000 ± 8,000	11,000 ± 1,500	3.8
	Aspartate	190,000 ± 24,000	99,000 ± 13,000	1.9
	Glutamine	1,600,000 ± 300,000	430,000 ± 64,000	3.7
	Cycloleucine	1,100,000 ± 150,000	370,000 ± 36,000	3.0
	Buffer control	3,200 ± 930	2,700 ± 400	1.2
Aspartate	Malate	66,000 ± 6,800	70,000 ± 13,000	0.9
	Succinate	86,000 ± 21,000	101,000 ± 6,500	0.9
	Aspartate	330,000 ± 29,000	49,000 ± 5,100	6.7
	Glutamine	1,000,000 ± 150,000	99,000 ± 31,000	10
	Cycloleucine	680,000 ± 95,000	82,000 ± 14,000	8.3
	Buffer control	9,500 ± 1,900	17,000 ± 1,600	0.6
Glutamine	Malate	42,000 ± 3,000	53,000 ± 7,400	0.8
	Succinate	60,000 ± 7,100	47,000 ± 9,800	1.3
	Fumarate	34,000 ± 2,500	40,000 ± 6,200	0.9
	Aspartate	74,000 ± 7,200	13,000 ± 1,400	5.7
	Glutamine	690,000 ± 180,000	9,200 ± 2,100	75
	Cycloleucine	570,000 ± 80,000	34,000 ± 5,600	17
	Buffer control	5,000 ± 450	4,300 ± 530	1.2
Cycloleucine	Succinate	32,000 ± 4,000	86,000 ± 14,000	0.4
	Aspartate	94,000 ± 9,400	34,000 ± 3,000	2.8
	Glutamine	665,000 ± 46,000	39,000 ± 4,800	17
	Cycloleucine	570,000 ± 85,000	49,000 ± 3,800	12
	Buffer control	5,100 ± 600	5,100 ± 300	1.0

[a] The amounts of attractant per competitor are as follows: malate, succinate, fumarate, and aspartate, 50 mM; glutamine and cycloleucine, 10 mM.

[b] Values are averages of two separate trials, each performed in quadruplicate.

[c] Ratio of mean cells per capillary for cells in buffer versus cells in buffer plus competitor.

the presence of aspartate. These results seem to indicate that aspartate, glutamine, and cycloleucine compete with one another, presumably for the same chemoreceptor, and that taxis to succinate and malate is mediated by a second receptor.

Glutamine strongly inhibited taxis to both aspartate and cycloleucine, while chemotaxis to dicarboxylic acids was not inhibited by glutamine. In reciprocal experiments, the addition of 10 mM cycloleucine to the suspension of bacteria in the wells strongly inhibited taxis to aspartate and glutamine but enhanced chemotaxis to succinate (Table 2). These findings agree with the aspartate competition results, indicating that there is a single receptor for aspartate, glutamine, and cycloleucine and there is an independent receptor for the dicarboxylic acids.

Microscopic examination indicated that there were immediate (2- to 5-s) increases in smooth swimming behavior of *R. meliloti* upon the addition of 10 mM glutamine or cycloleucine. Adaptation, evidenced by a return to normal swim-tumble switching frequency, was observed roughly 10 min after the addition of glutamine and about 15 min after the addition of cycloleucine. Cells preincubated with cycloleucine for 5 to 15 min showed a decrease in smooth swimming responsiveness to glutamine. Adaptation times were variable and difficult to assess. It was not possible to determine visually whether C_4 dicarboxylates or aspartate induced changes in swimming behavior or adaptation in *R. meliloti* JJ1c10.

C_4 dicarboxylate chemotaxis in *dct* mutants. The *dctA* mutant (*R. meliloti* 4F6) was less responsive to succinate than the wild type, but the differences were small and appeared to be dependent on succinate concentration (Table 3). Substantial reductions in responsiveness were seen only

TABLE 3. Comparison of the chemotactic responses of *R. meliloti* wild-type and *dct* mutant strains to succinate, aspartate, and glutamine

Attractant	Mean no. of cells/capillary ± SD[a]		Response ratio[b]
	Wild type	Mutant	
Succinate	JJ1c10	4F6 (*dctA*::Tn*5*)	
100 mM	83,000 ± 15,000	62,000 ± 12,000	1.3
50 mM	79,000 ± 15,000	50,000 ± 2,800	1.6
Aspartate (10 mM)	94,000 ± 9,100	79,000 ± 5,500	1.2
Buffer control	8,000 ± 820	7,400 ± 2,800	1.1
Succinate	JJ1c10	R639 (*dctB*::Tn*5*)	
100 mM	36,000 ± 200	40,000 ± 2,400	0.9
50 mM	52,000 ± 8,300	28,000 ± 4,000	1.9
Aspartate (100 mM)	180,000 ± 42,000	140,000 ± 19,000	1.3
Buffer control	6,500 ± 200	4,500 ± 900	1.4
Succinate	1021	F26 (*dctABD* deletion)	
100 mM	62,000 ± 5,000	34,000 ± 3,900	1.8
50 mM	49,000 ± 7,700	49,000 ± 8,900	1.0
Aspartate			
100 mM	235,000 ± 25,000	53,000 ± 9,000	4.4
50 mM	236,000 ± 32,000	143,000 ± 10,000	1.7
Glutamine (10 mM)	680,000 ± 55,000	590,000 ± 52,000	1.2
Buffer control	6,000 ± 900	6,100 ± 1,100	1.0

[a] Values are averages from two separate trials, each performed in quadruplicate.

[b] Ratio of mean cells per capillary for wild-type versus *dct* mutant.

TABLE 4. Comparison of the chemotactic responses of *R. meliloti* wild-type and *rpoN* mutant strains to succinate, aspartate, and glutamine

Attractant	Mean no. of cells/capillary ± SD[a]		Response ratio[b]
	Wild type	*rpoN*	
Aspartate			
100 mM	380,000 ± 29,000	53,000 ± 12,000	7.2
50 mM	400,000 ± 41,000	150,000 ± 34,000	2.7
10 mM	150,000 ± 11,000	140,000 ± 7,700	1.1
5 mM	73,000 ± 3,900	87,000 ± 7,900	0.8
Succinate			
100 mM	95,000 ± 7,000	95,000 ± 8,000	1.0
50 mM	80,000 ± 10,000	150,000 ± 8,000	0.5
10 mM	30,000 ± 3,000	58,000 ± 9,000	0.5
Glutamine (10 mM)	900,000 ± 20,000	1,000,000 ± 150,000	0.9

[a] Values are averages from two separate trials, each performed in quadruplicate.

[b] Ratio of mean cells per capillary for wild-type versus *rpoN* mutant.

at 50 mM succinate. Similar results were obtained with the *dctB* mutant (Table 3). It was not possible to test the *dctD* mutant in these assays because of its slow growth and poor motility. Strain F726, a *dctABD* deletion mutant derived from wild-type strain 1021, was significantly less responsive to succinate at concentrations of 100 mM but not 50 mM (Table 3).

Considerably greater differences in the chemotactic responses of the F726 deletion mutant were seen with aspartate. This mutant was two- to fourfold less responsive than its parent to 50 and 100 mM aspartate, even though it was just as responsive to glutamine.

Effects of mutations in *rpoN* on chemotaxis to C_4 dicarboxylic acids. Comparison of the *R. meliloti* 1021 wild type with an *rpoN* mutant derivative showed differences in chemotactic responses to both aspartate and succinate (Table 4). Chemotaxis to succinate increased ca. twofold in the *rpoN* mutant at 10 and 50 mM but was unchanged at 100 mM. The responses of the *rpoN* mutant to aspartate were only 40 and 15% of the wild-type responses at 50 and 100 mM, respectively, but were unchanged at 5 and 10 mM. Responses to glutamine were unaltered in the *rpoN* mutant. These results indicate that the alternative sigma factor *rpoN* may play a significant role in regulating chemotaxis to aspartate but has little if any effect on taxis to succinate or glutamine, at least under the culture conditions examined.

Cycloleucine as an inhibitor of methylation in *R. meliloti* chemotaxis. In initial studies to determine whether protein methyl esterification is involved in *R. meliloti* chemotaxis, as it is in *E. coli* (21, 38, 42), we examined the effects of cycloleucine on the chemotactic responsiveness, swimming behavior, and adaptation of strain JJ1c10. Cycloleucine is a methionine analog which inhibits synthesis of *S*-adenosyl-L-methionine, and hence methylation, in *E. coli*. *E. coli* cells treated with cycloleucine behave the same as methionine-starved cells (3). Methionine starvation results in loss of the adaptation response in *E. coli* and *Bacillus subtilis*, causing abnormally long periods of smooth swimming and loss of chemotactic responsiveness, as measured by capillary assays (28, 41, 42). Surprisingly, cycloleucine was shown to be a potent attractant for *R. meliloti* (Table 1). Although the addition of cycloleucine to suspensions of *R. meliloti* resulted in an increase in smooth swimming behavior, there was no corresponding general loss of chemotactic responsiveness. Because conclusions regarding the role of methylation in chemotaxis of *R. meliloti* could not be drawn from these experiments with cycloleucine, in vivo methylation and methanol release techniques were used instead.

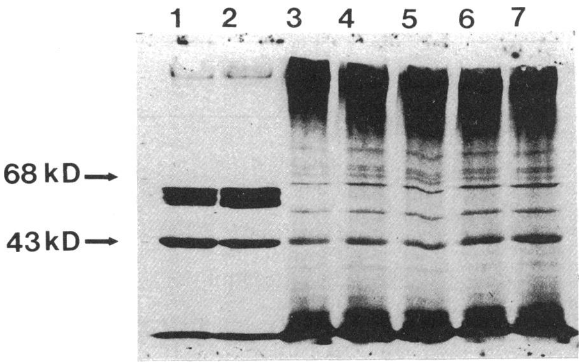

FIG. 2. In vivo methylation of *R. meliloti* JJ1c10 and *E. coli* Rp437 proteins. Cells were treated with chloramphenicol and incubated with L-[*methyl*-^{3}H]methionine for 60 min. Buffer or attractant was added 5 min before the methylation reaction was stopped. Samples were subjected to SDS-PAGE, and a fluorogram of the gel was prepared. Lanes: 1 and 2, *E. coli* RP437 (1, buffer; 2, 5 mM aspartate); 3 through 7, *R. meliloti* JJ1c10 (3, buffer; 4, 5 mM cycloleucine; 5, 5 mM glutamine; 6, 5 mM aspartate, 7, 5 mM succinate). Equivalent amounts of protein were present in each well.

In vivo methyl esterification of proteins. *E. coli* and *R. meliloti* were incubated with L-[*methyl*-^{3}H]methionine in the presence of chloramphenicol, with and without chemoattractants, in order to determine whether any proteins showed evidence of increased methyl esterification corresponding to the methylation of MCPs in response to attractants. The results are illustrated in Fig. 2. *E. coli* cells showed substantial incorporation of label into proteins of ca. 43,000 and 55,000 Da. After a 5-min incubation of *E. coli* with the attractant aspartate, there was a twofold increase in the label incorporated into the protein in the 55,000-Da range, consistent with previously reported increases in methyl esterification of MCPs in this organism (8, 21). No equivalent increase in incorporation of label was observed in *R. meliloti* after exposure to either strong attractants such as cycloleucine and glutamine or moderate attractants aspartate and succinate. Similar experiments with *R. meliloti* cells stimulated for various times and at different concentrations of glutamine also failed to show detectable increases in methylation. The level of label incorporated into *R. meliloti* proteins varied from experiment to experiment, with most of the label running close to the tracking dye. The total amount of protein in each of the wells in Fig. 2 was roughly equivalent.

Methanol release. The methanol release-diffusion assay described by Chelsky and Dahlguist (5) was also used to detect methyl esterification of proteins in *R. meliloti* in parallel with the in vivo methylation experiments described above. A three- to fourfold increase in release of labeled methanol was observed for *E. coli* cells exposed to 5 mM aspartate (Fig. 3A). A shift in mobility of the methyl esterified proteins was also observed. This is in good agreement with results from the in vivo methylation experiments (Fig. 2). In previous studies, multiple banding patterns of *E. coli* MCPs on SDS-polyacrylamide gels have been shown to correspond to various levels of methylation (8).

In contrast, *R. meliloti* cells stimulated with cycloleucine, aspartate, or succinate showed, if anything, a decrease in

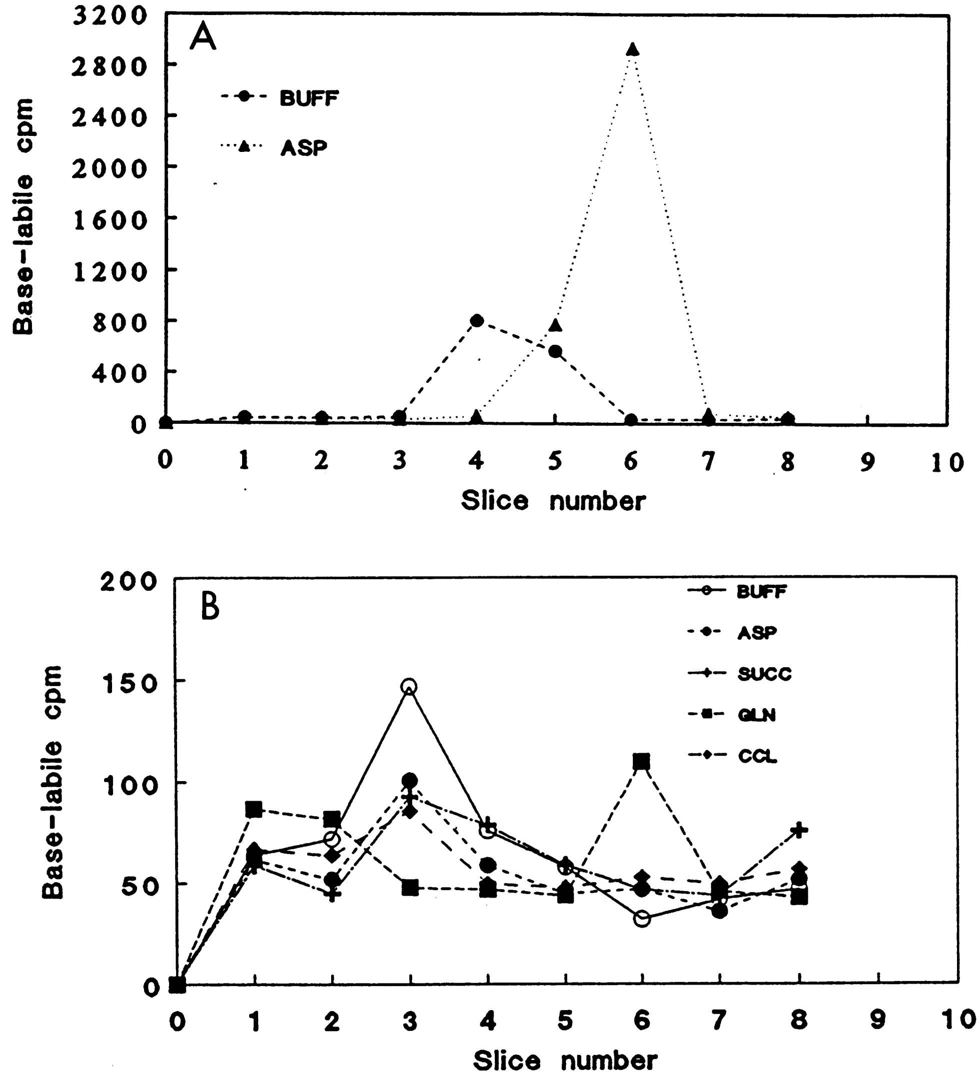

FIG. 3. Volatile, base-labile radioactivity from protein-methyl esters of *E. coli* RP437 and *R. meliloti* JJ1c10 labelled with L-[*methyl*-^{3}H]methionine. (A) Methanol release in response to buffer (BUFF) or aspartate (ASP) in *E. coli* RP437. (B) Methanol release in response to buffer (BUFF), aspartate (ASP), succinate (SUCC), glutamine (GLN), or cycloleucine (CCL) in *E. coli* JJ1c10. In both panels A and B, the region of the gel between 43 and 94 kDa was analyzed. Background levels of radioactivity (19 cpm) were not subtracted.

methanol release in relation to cells exposed to buffer alone (Fig. 3B). A small increase in methanol release was observed for cells stimulated with 5 mM glutamine, peaking in slice 6 (ca. 55,000 Da). This corresponds to a 3.4-fold increase in methanol release compared with that of control cells in this molecular weight range. However, it is slightly less than the peak level of methanol released by control cells observed in slice 3 (ca. 68,000 Da).

DISCUSSION

The present results show that chemotaxis of *R. meliloti* to the C_4 dicarboxylic acids is essentially independent of transport or metabolism of these compounds. Because mutations in the *dctB* gene did not abolish chemotaxis to C_4 dicarboxylic acids, *R. meliloti* must have two separate receptors or sensory mechanisms for detecting C_4 dicarboxylic acids in the environment, one (as yet unidentified) that initiates chemotactic responses and another (*dctB*) that regulates transport-related gene expression. Furthermore, because mutations in *dctA* had little effect on taxis to the C_4 acids, chemotaxis to the C_4 acids in *R. meliloti* does not require active transport, as does taxis to propionic acid in *Rhodobacter* species and adipic acid in *Pseudomonas* species (20, 40). Subsequent metabolism of dicarboxylic acids does not seem to be required for chemotactic responses either, since itaconic acid, a nonmetabolizable analog of succinate which is transported by the Dct system (32) serves as a chemoattractant on the same order as succinate (Table 1). Also, chemotaxis to succinate was not measurably induced by growth on succinate in this strain, in contrast to transport activity.

While these results clearly establish the basic independence of the C_4 dicarboxylic acid taxis and transport mechanisms, there is nonetheless evidence for some manner of interconnection between taxis and transport. Mutations in the *dctA* and *dctB* genes both resulted in significant (ca. 30%)

reductions in chemotactic responses to succinate (Table 3), even though these two genes are not required for chemotaxis and have dissimilar functions. The magnitude of the *dct* gene effect on taxis was dependent on the concentration of succinate in the capillary tubes. This may indicate that the chemotactic responsiveness of the bacteria to C_4 dicarboxylic acids changes in response to Dct activity; i.e., the chemotactic response curve shifts to lower succinate concentrations when the transport system is active, making the bacterium more responsive, and shifts to higher succinate concentrations in the absence of transport activity.

The Dct system and chemotaxis to aspartate are more clearly linked. Disruption of *dctA* or *dctB* in strain JJ1c10 had little or no effect on aspartate chemotaxis, although expression of these genes is required for uptake of aspartate (46). Thus, aspartate taxis does not require transport. However, the deletion of all three *dct* genes, *dctA*, *dctB*, and *dctC*, substantially diminished taxis to aspartate at both 100 and 50 mM (Table 3). If strain background can be ignored, this result implies that taxis to aspartate is quite dependent on *dctD*. Mutation of *rpoN* likewise resulted in similar reductions in chemotactic responsiveness to high concentrations of aspartate (Table 4). *dctD* and *rpoN* act jointly to initiate transcription of the DctA transport protein in *R. meliloti* (36, 46). It therefore seems likely that *dctD* and *rpoN* also control transcription of one or more genes involved in, but not essential for, aspartate chemotaxis.

From competition experiments, the C_4 dicarboxylic acids appear to share one common chemoreceptor, while the amino acids aspartate, glutamine, and cycloleucine share a second, independent chemoreceptor (Table 2). Malate appears to have a more general effect.

A crucial part of behavioral responses to chemoattractants is the process of adaptation, i.e., the return to prestimulus behavior. The process of adaptation makes it possible for the organism to respond to other attractants or environmental changes. Behavioral adaptation involves the reversible methyl esterification of chemoreceptor proteins in a wide range of bacterial genera (38), apparently including *Rhizobium* (2). Our microscopic examinations indicate that *R. meliloti* does adapt to strong chemotactic stimuli within 10 to 15 min. In contrast, *E. coli* adapts to strong attractants within a range of a few seconds to a couple of minutes, depending on the attractant concentration (21, 38, 42). These differences in adaptation time may reflect differences in the molecular mechanisms of adaptation.

Cycloleucine is a methionine analog which inhibits behavioral adaptation and chemotactic migration in *E. coli* by preventing methyl esterification of MCPs (3). In *R. meliloti* JJ1c10, however, cycloleucine proved to be a very potent chemoattractant, with a threshold concentration of about 2×10^{-7} M. We do not know whether cycloleucine acts to block protein methylation in rhizobia as it does in *E. coli*, but it did affect chemotaxis of *R. meliloti* to various compounds. The presence of cycloleucine did not appear to affect general motility, but it significantly enhanced chemotaxis to succinate (Table 2). In contrast, cycloleucine strongly inhibited chemotaxis to both glutamine and aspartate. This inhibition might result from a blockage of receptor methylation, as in *E. coli*, or might reflect the existence of a common chemoreceptor for glutamine, aspartate, and cycloleucine. This latter possibility is strengthened by the strong reciprocal inhibition of taxis to cycloleucine by added glutamine. The positive effect of cycloleucine on chemotaxis to succinate has no ready explanation but is further evidence that C_4 dicarboxylates and glutamine do not share a common chemoreceptor.

In vivo methylation and methanol release assays gave the expected results for *E. coli* (5, 21) but failed to show a significant increase in methylation in *R. meliloti* cells exposed to any of the attractants tested. The low level of protein methylation in *R. meliloti* may have one or more explanations. First, *R. meliloti* may have significantly fewer MCP-like receptor proteins per cell than *E. coli*, making changes in methyl esterification too small to detect. Second, methylation may not significantly change the SDS-PAGE mobility of MCP-like proteins in this bacterium, making it more difficult to detect changes in the degree of methyl esterification. Third, attractants may cause a high turnover of methyl groups on the MCPs, such as that in *B. subtilis* (16), in which case the methyl groups stay in the system rather than evolving directly as methanol. The methods used in the present study were not appropriate for the detection of methyl group turnover, making this an obvious next step. Finally, *R. meliloti* may not use methylation of chemoreceptors as a significant mechanism of adaptation, or it may use a reverse methylation system, i.e., decreased methylation in response to an attractant. None of these possibilities rules out MCP-like chemoreceptor proteins with homology to *E. coli* MCPs. It should be noted that the chemosensory system of *R. sphaeroides* does not involve an MCP system, while *Rhodobacter rubrum* does have an MCP-based chemosensory system (40), providing an example of two closely related bacteria which possess decidedly different chemotaxis systems. Similar differences between *R. leguminosarum* (2) and *R. meliloti* may exist.

In preliminary experiments, Western blots (immunoblots) of proteins from three different *R. meliloti* strains with *E. coli* anti-Tar antibodies gave evidence of a weakly cross-reactive doublet of ca. 68 kDa. The limited reaction of *R. meliloti* proteins with *E. coli* anti-Tar antibody relative to reactions with the *E. coli* proteins may be due to limited homology, a low number of available antigenic sites, or a low copy number. Further experiments with affinity-purified anti-Tar antibodies and membrane fractions are needed to determine whether *R. meliloti* does indeed have proteins which are structurally, and perhaps functionally, related to the chemoreceptor MCPs of enteric bacteria.

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