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# Functional Analysis of Nine Putative Chemoreceptor Proteins in *Sinorhizobium meliloti*[▽]†

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**The genome of the symbiotic soil bacterium *Sinorhizobium meliloti* contains eight genes coding for methyl-accepting chemotaxis proteins (MCPs) McpS to McpZ and one gene coding for a transducer-like protein, IcpA. Seven of the MCPs are localized in the cytoplasmic membrane via two membrane-spanning regions, whereas McpY and IcpA lack such hydrophobic regions. The periplasmic regions of McpU, McpV, and McpX contain the small-ligand-binding domain Cache. In addition, McpU possesses the ligand-binding domain TarH. By probing gene expression with *lacZ* fusions, we have identified *mcpU* and *mcpX* as being highly expressed. Deletion of any one of the receptor genes caused impairments in the chemotactic response toward most organic acids, amino acids, and sugars in a swarm plate assay. The data imply that chemoreceptor proteins in *S. meliloti* can sense more than one class of carbon source and suggest that many or all receptors work as an ensemble. Tactic responses were virtually eliminated for a strain lacking all nine receptor genes. Capillary assays revealed three important sensors for the strong attractant proline: McpU, McpX, and McpY. Receptor deletions variously affected free-swimming speed and attractant-induced chemokinesis. Noticeably, cells lacking *mcpU* were swimming 9% slower than the wild-type control. We infer that McpU inhibits the kinase activity of CheA in the absence of an attractant. Cells lacking one of the two soluble receptors were impaired in chemokinetic proficiency by more than 50%. We propose that the internal sensors, IcpA and the PAS domain containing McpY, monitor the metabolic state of *S. meliloti*.**

The process of chemotaxis enables many motile bacterial species to sense their environment and move in a beneficial direction. The underlying signaling pathway for responding to changes in the concentrations of chemical attractants or repellents has been most intensely studied for *Escherichia coli* and *Salmonella enterica* serovar Typhimurium (for reviews, see references 24 and 69). In the absence of a chemical stimulus, *E. coli* shows a random swimming pattern consisting of alternating runs and tumbles. The addition of an attractant or the removal of a repellent promotes counterclockwise flagellar rotation and therefore straight runs. Ergo, the cell is directed to a more advantageous environment. The signal transduction pathway to the flagellar motor consists of chemoreceptor proteins and a two-component signaling system. *E. coli* uses four membrane-bound methyl-accepting chemotaxis proteins (MCPs)—Tar for aspartate and maltose, Tsr for serine, Trg for ribose and galactose, and Tap for dipeptides—as well as the membrane-bound Aer as an oxygen sensor (16, 24). MCP molecules typically consist of a periplasmic ligand-binding domain, two transmembrane helices, and a highly conserved cytoplasmic signaling domain (24, 67). To enable high sensitivity over a range of attractant concentrations, adaptational modifications are introduced at specific glutamate residues in two methylation helices, MH1 and MH2 (38). Methyl groups are transferred from *S*-adenosylmethionine by the methyltransferase CheR (72), while their removal is accomplished by the methylesterase CheB (33, 68). The highly abundant major receptors in *E. coli*, Tsr and Tar, have an NWETF pentapeptide sequence at the C terminus, which serves as a docking site for CheR and CheB (12, 25, 77).

Recent studies suggest that chemotaxis in other bacteria departs from the *E. coli* model by involving more *che* genes and chemoreceptors (4, 7, 17, 59, 70). The nitrogen-fixing plant symbiont *Sinorhizobium meliloti*, a member of the alpha subgroup of proteobacteria (52), differs from the enterobacterial (gamma-subgroup) behavioral scheme in its modes of flagellar rotation, signal processing, and gene regulation (59). The rigid complex flagellar filaments consist of four related flagellin subunits, and interflagellin bonds lock the filaments into right-handedness (21, 29, 60). Hence, *S. meliloti* cells are propelled by flagella that rotate exclusively clockwise, and swimming cells respond to tactic stimuli by modulating their rotary speed (8, 58). In *E. coli*, tactic signals are processed by a single response regulator, CheY, and a phosphatase, CheZ. In contrast, signal processing in *S. meliloti* involves a retrophosphorylation loop with two response regulators, CheY1 and CheY2, but no phosphatase (64, 65). CheY2 is the main regulator of motor function, causing a decrease in the rotary speed of the unidirectional clockwise-rotating flagellar motor (59). It has been reported previously that *S. meliloti* exhibits positive chemotactic responses toward a wide range of substances such as amino acids, sugars, and exudates from roots of legume host plants (20, 22, 29, 32, 45). *S. meliloti* has nine putative chemoreceptors to sense the concentrations of these attractants (26, 48). In order to elucidate the roles of individual chemoreceptor proteins in chemotaxis, we introduced single and multiple gene

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† Dedicated to Rüdiger Schmitt on the occasion of his 70th birthday.

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TABLE 1. Bacterial strains and plasmids

| Strain or plasmid | Relevant characteristic(s)[a,b] | Source or reference |
|---|---|---|
| Strains | | |
| *E. coli* | | |
| DH10B | *recA1 endA1* | GIBCO/BRL |
| S17-1 | *recA endA thi hsdR* RP4-2 Tc::Mu::Tn*7* $Tp^r$ $Sm^r$ | 62 |
| *S. meliloti* | | |
| RU11/001 | $Sm^r$; spontaneously streptomycin resistant wild-type strain | 53 |
| RU11/310 | $Sm^r$ Δ*cheA* | 64 |
| RU11/803 | $Sm^r$ Δ*mcpW* | This work |
| RU11/804 | $Sm^r$ Δ*mcpY* | This work |
| RU11/805 | $Sm^r$ Δ*mcpX* | This work |
| RU11/815 | $Sm^r$ Δ*icpA* | This work |
| RU11/818 | $Sm^r$ Δ*mcpZ* | This work |
| RU11/828 | $Sm^r$ Δ*mcpU* | This work |
| RU11/830 | $Sm^r$ Δ*mcpV* | This work |
| RU11/838 | $Sm^r$ Δ*mcpT* | This work |
| RU13/107 | $Sm^r$ Δ*mcpY* Δ*icpA* | This work |
| RU13/148 | $Sm^r$ Δ*mcpS* (ΔSMa1556) | This work |
| RU13/149 | $Sm^r$ Δ*mcpS* (ΔSMa1556) Δ*mcpT*Δ*mcpU*Δ*mcpV*Δ*mcpW*Δ*mcpX*Δ*mcpY* Δ*mcpZ*Δ*icpA* (Δ9) | This work |
| Plasmids | | |
| pK18*mobsacB* | $Km^r$ *lacZ mob sacB* | 57 |
| pPHU234[c] | $Tc^r$; promoterless *lacZ* fusion | 36 |
| pRU2250 | $Tc^r$; *icpA*(1,974 bp)-*lacZ* (*che*) fusion cloned into pPHU235 | 63 |
| pRU2283 | $Tc^r$; *mcpU*(456 bp)-*lacZ* fusion cloned into pPHU236 | This work |
| pRU2782 | $Tc^r$; *mcpT*(320 bp)-*lacZ* fusion cloned into pPHU235 | This work |
| pRU2783 | $Tc^r$; *mcpV*(415 bp)-*lacZ* fusion cloned into pPHU236 | This work |
| pRU2784 | $Tc^r$; *mcpW*(303 bp)-*lacZ* fusion cloned into pPHU236 | This work |
| pRU2787 | $Tc^r$; *mcpZ*(409 bp)-*lacZ* fusion cloned into pPHU236 | This work |
| pRU2898 | $Tc^r$; *mcpY*(786 bp)-*lacZ* fusion cloned into pPHU236 | This work |
| pRU2899 | $Tc^r$; *mcpS*(869 bp)-*lacZ* (*che2*) fusion cloned into pPHU236 | This work |
| pRU2994 | $Tc^r$; *mcpX*(590 bp)-*lacZ* fusion cloned into pPHU236 | This work |

[a] Nomenclature according to Bachmann (9) and Novick et al. (51).
[b] For the selection of the $Km^r$ marker in *S. meliloti*, neomycin was used at 120 mg/liter.
[c] pPHU235 and pPHU236 are pPHU234 derivatives with different translational phasing of *lacZ* (36).

disruptions and analyzed the chemotactic abilities of the resulting mutant strains toward nutrients.

## MATERIALS AND METHODS

**Bacterial strains and plasmids.** Derivatives of *E. coli* K-12 and *S. meliloti* MV II-1 (37) and the plasmids used are listed in Table 1.

**Media and growth conditions.** *E. coli* strains were grown in Luria broth (43) at 37°C. *S. meliloti* strains were grown in TYC (0.5% tryptone, 0.3% yeast extract, 0.13% $CaCl_2 \cdot 6H_2O$ [pH 7.0]) at 30°C (60). Motile cells prepared for swimming velocity analysis and capillary assays were grown for 2 days in TYC with streptomycin, diluted first 1:5 in 3 ml of TYC with streptomycin for 24 h and then 1:500 in 10 ml RB minimal medium (29), layered on Bromfield agar plates (64), and incubated at 30°C for 15 h to an optical density at 600 nm ($OD_{600}$) of 0.1. The following antibiotics were used at the indicated final concentrations: for *E. coli*, kanamycin at 50 mg/liter and tetracycline at 10 mg/liter; for *S. meliloti*, neomycin at 120 mg/liter, streptomycin at 600 mg/liter, and tetracycline at 10 mg/liter.

**Swarm assays.** Swarm plates containing Bromfield medium and 0.3% Bacto agar and swarm plates containing RB minimal medium complemented with a carbon source at a concentration of $10^{-4}$ M and 0.27% Bacto agar were inoculated with 3-μl droplets of the test culture and incubated at 30°C for 3 to 4 days.

**Computerized motion analysis of free-swimming cells.** The speed of free-swimming cells at an $OD_{600}$ of 0.1 was measured by using the computerized motion analysis of the Hobson BacTracker system (Hobson Tracking Systems, Sheffield, United Kingdom) as previously described (64). Cells were observed with a Zeiss standard 14 phase-contrast microscope (magnification, ×400) at a constant room temperature of 22°C. The effects of chemoattractants on the motile behavior of wild-type and mutant cell populations were determined within 20 s of their addition to the cell samples.

**Capillary assays.** Capillary assays were performed essentially as described by Adler (2) with minor modifications according to Götz et al. (30). Cells grown to an $OD_{600}$ of 0.1 were centrifuged at 2,000 × *g* for 5 min at room temperature and resuspended in RB minimal medium without a carbon source to an $OD_{600}$ of 0.1. Closed U-shaped tubes (bent from 65-mm micropipettes; Drummond Scientific Co., Broomall, PA) were placed between two glass plates and filled with 0.4 ml of the bacterial suspension. Capillary tubes (1-μl disposable micropipettes; DESAGA GmbH, Wiesloch, Germany) were sealed at one end and filled with an attractant dissolved in RB minimal medium. The capillaries were inserted, open end first, into the bacterial pond and incubated for 2 h in a thermostat chamber at 30°C. Capillaries were removed, the sealed end was cut off, and the complete contents were transferred to 1 ml RB minimal medium. Dilutions were plated in duplicate on TYC plates containing streptomycin. After incubation for 3 days at 30°C, colonies were counted.

**Genetic manipulations and reporter gene assay.** Deletion mutants of *S. meliloti* (listed in Table 1) were generated in vitro by overlap extension PCR as described by Higuchi (34). Constructs containing the mutation were cloned into the mobilizable suicide vector pK18*mobsacB*, used to transform *E. coli* S17-1, and conjugally transferred to *S. meliloti* by filter matings according to the method of Simon et al. (62). Allelic replacement was achieved by sequential selections on neomycin and 10% sucrose as described previously (64). Confirmation of allelic replacement and elimination of the vector was obtained by PCR with gene-specific primers, DNA sequencing, and Southern blotting. The broad-host-range plasmid pPHU234 and its derivatives pPHU235 and pPHU236 served as vectors for translational fusions of the seven *mcp* promoters and the promoters of the two *che* operons. The resulting *lacZ* fusion plasmids were used to transform *E. coli* S17-1 and then were transferred conjugally to RU11/001 by streptomycin-tetracycline double selection, as described by Labes et al. in 1990 (41).

**DNA methods.** *S. meliloti* DNA was isolated and purified as described previously (64). Plasmid DNA was purified with NucleoSpin (Macherey Nagel, Düren,

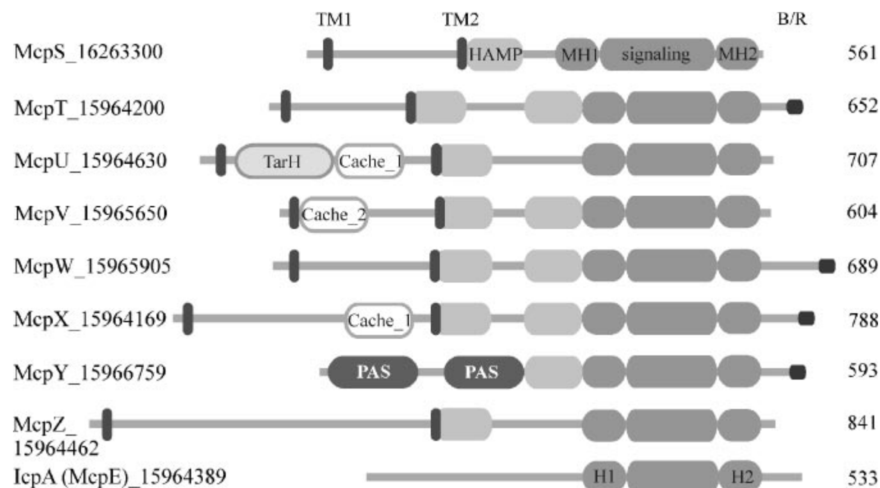


FIG. 1. Domain organization of chemoreceptor proteins from *S. meliloti* according to the MiST (73), Pfam (15), and SMART (42) databases. GI (gene identification) numbers for each protein are listed after the name. Conserved domains are symbolized by identical shading. TM1 and TM2, transmembrane regions 1 and 2; HAMP, conserved signal transduction domain in histidine kinases, adenylyl cyclases, methyl-accepting chemotaxis proteins, and phosphatases (6); H1 and H2, helices 1 and 2 lacking the conserved methylation sites in IcpA; signaling, MCP signaling domain and interaction site for CheW and CheA; B/R, interaction site for CheB and CheR; TarH, four-helix ligand-binding domain (74); Cache, acronym formed from the names of proteins in which these signaling domains were recognized (animal $Ca^{2+}$ channel subunits and prokaryotic chemotaxis receptors) (5); PAS, acronym formed from the names of the proteins in which imperfect repeat sequences were first recognized (the *Drosophila* period clock protein [PER], the vertebrate aryl hydrocarbon receptor nuclear translocator [ARNT], and the *Drosophila* single-minded protein [SIM] (49, 54).

Germany), and DNA fragments or PCR products were purified from agarose gels using a QiaEx DNA purification kit (QIAGEN, Hilden, Germany) and a GFX PCR and gel band purification kit (Amersham Biosciences). PCR amplification of chromosomal DNA and Southern blotting were carried out according to published protocols (66).

**β-Galactosidase assays.** Cultures of *S. meliloti* containing *lacZ* fusions were sampled, diluted 1:1 in Z buffer (46), permeabilized with 1 drop of toluene, and assayed for β-galactosidase activity by the method of Miller (46).

## RESULTS

***The S. meliloti* genome contains eight *mcp* genes and one atypical chemoreceptor gene.** The chemotaxis operon (*che* operon) of *S. meliloti* is composed of the *orf1*, *orf2*, *cheY1*, *cheA*, *cheW*, *cheR*, *cheB*, *cheY2*, *cheD*, and *orf10* genes (31, 66). The gene product of *orf1* has been classified as a transducer-like protein based on the presence of a signaling domain homologous to *E. coli* MCPs (24, 59). However, the absence of Glu or Gln residues that would serve as methyl-accepting sites, as well as the lack of hydrophobic transmembrane and periplasmic receptor regions, prompted us to name this atypical chemoreceptor IcpA (for internal chemotaxis protein A) (59). IcpA is also listed as McpE of *S. meliloti* strain 1021 in GenBank. PCR analysis using degenerate primers flanking the conserved signaling domain and Southern blot hybridization combined with a plasmid rescue approach were performed to identify seven chemoreceptor genes in the chromosome. They were named *mcpT*, *mcpU*, *mcpV*, *mcpW*, *mcpX*, *mcpY*, and *mcpZ*, since their gene products exhibit typical features of *E. coli* MCPs (39, 48). The majority of these genes, except for *mcpW*, which is cotranscribed with a putative *cheW* gene, have a monocistronic organization and are scattered throughout the genome. The genome sequencing project of *S. meliloti* strain 1021 revealed the presence of an additional, eighth *mcp* gene, *mcpS*. It is localized on the symA plasmid and is organized as the third gene in a putative chemotaxis operon (the *che2* operon) containing *cheR*, *cheW*, *mcpS*, *cheA*, and *cheB* (14, 26).

The derived receptor polypeptide sequences yield proteins with molecular masses between 56 and 74 kDa. They can be classified as transmembrane and soluble receptors according to their domain organization. We utilized the MiST (microbial signal transduction) (73), Pfam (protein families database of alignments) (15), PSORTb (27), and SMART (simple modular architecture research tool) (42) databases for a complete domain architecture of all nine receptors as depicted in Fig. 1. Seven of the receptor proteins have two hydrophobic membrane-spanning (transmembrane) regions and are consequently localized in the cytoplasmic membrane with a large periplasmic sensing domain and a large cytoplasmic signaling domain. The sizes of the extracytoplasmic ligand-binding domains of McpT, McpV, and McpW are similar to that of the *E. coli* receptors Tar and Tsr, comprising 160 amino acid residues. In contrast, McpU, McpX, and McpZ are distinguished by extended periplasmic domains with 250 to 390 amino acid residues. The cytoplasmic domain of all MCPs contains the highly conserved region common to all chemosensory transducers across bacterial and archaeal species, consisting of a methylation helix (MH1), the signaling domain, and a second methylation helix (MH2) (69). Another conserved domain in MCPs is the HAMP (histidine kinases, adenylyl cyclases, MCPs, and phosphatases) domain, which is thought to participate in signal transmission from the periplasmic sensing domain to the cytoplasmic signaling domain of the transducer (18). In *S. meliloti*, a HAMP domain is present adjacent to the second transmembrane region of all transmembrane receptors. Five receptors have an additional HAMP domain in front of MH1 (Fig. 1).

In addition to the classical transmembrane receptors, *S. me-*

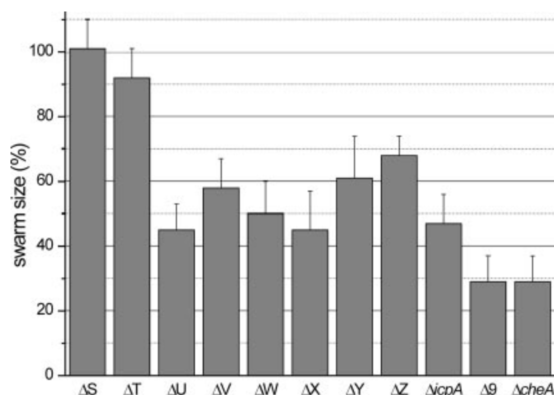


FIG. 2. Histogram of swarm sizes on Bromfield agar of 11 chemotaxis mutants relative to that of wild-type cells. Strain designations: ΔS to ΔZ, in-frame deletion of *mcpS* (RU13/148), *mcpT* (RU11/838), *mcpU* (RU11/828), *mcpV* (RU11/830), *mcpW* (RU11/803), *mcpX* (RU11/805), *mcpY* (RU11/804), and *mcpZ* (RU11/818), respectively; Δ*icpA*, in-frame deletion of *icpA* (RU11/815); Δ9, in-frame deletion of *mcpS*, *mcpT*, *mcpU*, *mcpV*, *mcpW*, *mcpX*, *mcpY*, *mcpZ*, and *icpA* (RU13/149); Δ*cheA*, in-frame deletion of *cheA* (RU11/310). Percentages of the wild-type swarm diameter (after subtraction of the 7-mm diameter of a nonmotile *fla* mutant) on 0.3% Bromfield agar are the means of 15 replicates.

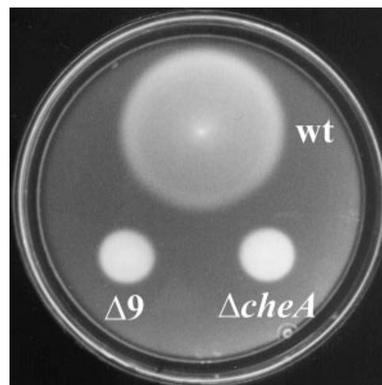


FIG. 3. Swarm test of wild-type *S. meliloti* RU11/001 (wt), the Δ9 receptor mutant RU13/149, and the *cheA* mutant RU11/310 (Δ*cheA*). Strains to be tested were transferred by micropipette (3 μl) onto Bromfield swarm plates and incubated at 30°C for 2 days. The diameter of a swarm ring reflects the motile and tactic proficiency of a given strain.

*liloti* possesses two receptors, McpY and IcpA, which lack obvious hydrophobic, membrane-spanning regions as predicted by the databases used in this study. This prediction gives rise to the suspicion that they are localized in the cytosol. McpY is distinguished by the presence of a tandem repeat of PAS domains in its N-terminal part (Fig. 1). PAS domains are known to function as sensors for oxygen, energy, and light (71). The signaling domain and the methylation helices, MH1 and MH2, including the position of the methylated sites, are highly conserved in McpY, whereas IcpA has only modest similarity to MH1 and MH2. Conspicuously, the methylated sites characteristic of classical MCPs are absent in both helices (H1 and H2) of IcpA. Nevertheless, the highly conserved signaling domain allows its classification as a transducer protein in chemotaxis. The Pfam data bank analysis also detected the presence of a Cache signaling domain, which is known to bind small molecules such as amino acids (5), in the periplasmic regions of McpU, McpV, and McpX. An additional conserved domain in the periplasmic region of McpU is TarH, homologous to the four-helix bundle, ligand-binding domains in *E. coli* transmembrane MCPs (74).

In *E. coli*, the high-abundance receptors, Tar and Tsr, differ from low-abundance receptors by the presence of a conserved carboxyl-terminal pentapeptide sequence, NWETF, that enhances adaptational covalent modification (11, 13). Four of the *S. meliloti* MCPs, namely, McpT (DWEEF), McpW (NWEEF), McpX (NWEEF), and McpY (DWENF), contain a modified NWETF motif with the consensus sequence (N/D)WEEF. Residues critical for the binding of CheR, namely, Trp in the second position and Phe in the fifth position, are conserved (61).

**Contribution of individual receptors to motility on Bromfield swarm plates.** Single chemoreceptor genes were deleted in frame by allelic exchange (57). An initial insight into the function of their gene products was provided by a comparison of swarm diameters obtained on Bromfield soft-agar plates with the wild-type standard (RU11/001). Unlike *E. coli*, which forms three distinct chemotactic bands on semisolid nutrient agar (1), *S. meliloti* does not form individual swarm rings. The formation of uniform swarm rings has also been reported for other bacterial species such as *Caulobacter crescentus* (23) and *Vibrio parahaemolyticus* (56). When motile proficiency was assessed, the Δ*mcpS* mutant showed no decrease in swarm size and the Δ*mcpT* mutant exhibited a decrease of only 8% in swarm diameter (Fig. 2). Three knockout mutants, the Δ*mcpV*, Δ*mcpY*, and Δ*mcpZ* mutants, generated swarms 55 to 70% of the wild-type swarm size. The greatest impairment was observed for strains lacking *mcpU*, *mcpW*, *mcpX*, or *icpA*, with a reduction in swarm size greater than 50%. In addition to the single gene disruptions, we deleted all nine receptor genes successively to create strain RU13/149, also named Δ9 (Table 1). When its behavior on Bromfield swarm plates was compared to that of the wild-type control (RU11/001), swarming was reduced by about 70%. A *cheA* deletion strain (RU11/310), which is chemotaxis deficient due to the lack of kinase activity, is similarly impaired (Fig. 3). Therefore, the phenotype of the Δ9 strain can be defined as nontactic.

**Sugars, amino acids, and organic acids provoke chemokinesis responses.** A multitude of compounds are released into the soil by plants, including numerous sugars, amino acids, and organic acids, which can be utilized as carbon sources by soil microorganisms (75). Previous studies reported positive chemotaxis of *S. meliloti* toward a number of organic compounds (20, 29, 45, 55). We tested the growth and swarming proficiencies of an *S. meliloti* wild-type strain (RU11/001) by using semisolid plates containing minimal medium with single carbon sources. Typically, swarming was optimal at concentrations of $10^{-4}$ M, whereas higher concentrations suppressed swarming. Organic acids of the tricarboxylic acid cycle (citrate, malate, succinate, and fumarate) were good carbon sources and attractants. Of the 20 L-amino acids tested, the formation of distinct swarm rings was observed only in the presence of glutamate, glutamine, histidine, lysine, or proline. We also

TABLE 2. Free-swimming speeds of the *S. meliloti* wild-type strain (RU11/001) without and with stimulation by chemoattractants

| Attractant[a] | Swimming velocity[b] (μm/s) | Chemokinesis (%)[c] |
|---|---|---|
| None | 36.9 | NA |
| Citrate | 38.5 | 4.3 |
| Fumarate | 38.4 | 4.1 |
| Malate | 38.4 | 4.1 |
| Succinate | 38.0 | 2.9 |
| Glutamate | 37.7 | 2.2 |
| Glutamine | 37.6 | 1.9 |
| Histidine | 39.0 | 5.7 |
| Lysine | 39.7 | 7.6 |
| Proline | 38.5 | 4.3 |
| Fructose | 38.7 | 4.9 |
| Galactose | 39.1 | 6.0 |
| Maltose | 39.4 | 6.8 |
| Mannitol | 38.3 | 3.8 |
| Saccharose | 39.3 | 6.5 |

[a] Attractants were diluted from concentrated stock solutions in RB minimal medium, buffered with 10 mM phosphate buffer (pH 7.0).

[b] Swimming velocity before and after stimulation with a chemoattractant ($10^{-4}$ M). For each strain, 1,000 individual tracks from five independent cell populations were analyzed by the Hobson BacTracker system.

[c] Given as the percentage of increase in swimming velocity after stimulation with a chemoattractant. NA, not applicable.

screened six D-sugars and one sugar alcohol, all of which served as good carbon sources and attractants.

As a response to attractant stimuli, *S. meliloti* increases its swimming speed, a phenomenon called chemokinesis (8, 64). To assess the potency of organic acids, amino acids, and sugars as attractants to induce chemokinesis, we used computerized motion analysis to monitor and average the free-swimming speeds of cell populations. Table 2 lists the free-swimming speeds of the *S. meliloti* wild-type strain (RU11/001) observed in the absence and the presence of chemoattractants. All organic acids except succinate were good attractants, eliciting an increase of about 4% in swimming speed. Responses to amino acids varied widely. Glutamate and glutamine were weak attractants, whereas histidine, lysine, and proline were potent attractants. All sugars were proven to provoke a very strong response (4 to 6.5%). This is in line with the findings of Malek (45), who reported that compound sugars were better chemoattractants than amino acids. However, other groups showed that sugars were weaker attractants than amino acids (20, 29). In essence, it is an extensive and problematic task to compare these studies, because different strains, cell culture growth conditions, and chemotaxis assays have been used.

**Contributions of individual receptors to motility on swarm plates with single carbon sources.** The swarming proficiencies of the wild-type strain, 10 receptor deletion strains, and a *cheA* deletion strain on organic acids (Fig. 4A), amino acids (Fig. 4B), and sugars (Fig. 4C) as sole carbon sources and attractants were analyzed. Swarm rings that formed on plates containing organic acids or amino acids had less-distinct outlines than those that formed on plates containing sugars. The blurred edges of swarms hampered analysis of swarm ring diameters. The Δ9 mutant strain (RU13/149), which lacks all nine receptor genes, and the *cheA* deletion strain were still able to generate swarms with 30 to 50% of the wild-type swarm

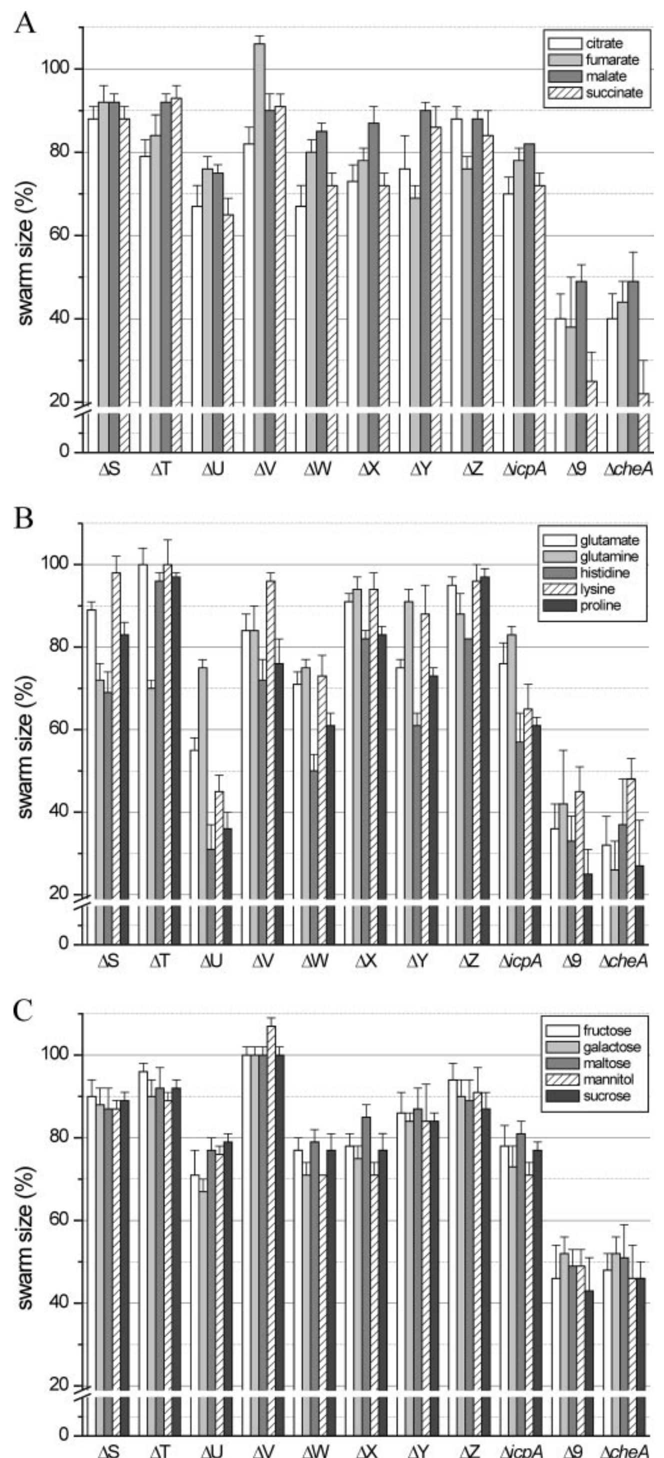


FIG. 4. Histogram of swarm sizes on single carbon sources of 11 chemotaxis mutants relative to that of wild-type cells. For strain designations, see the legend to Fig. 2. Shown are results for swarm behavior on organic acids (A), L-amino acids (B), and D-sugars (C). All carbon sources were used at a concentration of $10^{-4}$ M. Percentages of the wild-type swarm diameter (after subtraction of the 7-mm diameter of a nonmotile *fla* mutant) on 0.27% agar are the means of six replicates.

TABLE 3. Free-swimming speeds of the *S. meliloti* wild-type strain and 12 chemotaxis mutants without and with stimulation by proline

| Strain | Genotype | Swimming velocity (μm/s)[a] | | Chemokinesis (%)[b] |
|---|---|---|---|---|
| | | Without proline | With 10 mM proline | |
| RU11/001 | Wild type | 37.4 | 40.2 | 7.5 |
| RU13/148 | Δ*mcpS* | 37.5 | 40.2 | 7.2 |
| RU11/838 | Δ*mcpT* | 40.1 | 42.7 | 6.5 |
| RU11/828 | Δ*mcpU* | 33.9 | 36.7 | 8.3 |
| RU11/830 | Δ*mcpV* | 38.2 | 40.6 | 6.3 |
| RU11/803 | Δ*mcpW* | 37.9 | 40.1 | 5.8 |
| RU11/805 | Δ*mcpX* | 38.0 | 41.4 | 8.9 |
| RU11/804 | Δ*mcpY* | 36.5 | 37.4 | 2.5 |
| RU11/818 | Δ*mcpZ* | 37.1 | 40.0 | 7.8 |
| RU11/815 | Δ*icpA* | 38.5 | 39.8 | 3.4 |
| RU13/107 | Δ*mcpY* Δ*icpA* | 38.1 | 38.8 | 1.8 |
| RU13/149 | Δ9 (*icpASTUVWXYZ*) | 40.6 | 40.9 | 0.7 |
| RU11/310 | Δ*cheA* | 40.0 | 40.2 | 0.5 |

[a] Swimming velocity before and after stimulation with proline (10 mM). For each strain, 1,000 individual tracks from at least five independent cell populations were analyzed by the Hobson BacTracker system.

[b] Given as the percentage of increase in swimming velocity after stimulation with proline.

diameter, a behavior similar to that observed on Bromfield swarm plates (Fig. 2).

On swarm plates containing one of four different organic acids, most deletion strains generated swarms at 80 to 100% of the size of the wild-type swarm standard. Only strains with deletions in *mcpU*, *mcpW*, *mcpX*, and *icpA* formed swarms at 70 to 80% of the wild-type control size (Fig. 4A). Swarms generated on plates containing amino acids were more variable in size than those generated on organic acids or on sugars. Also noted as a result, swarm sizes were strongly reduced. In particular, swarms produced by strains lacking *mcpU*, *mcpW*, *mcpY*, and *icpA* were 30 to 75% of the wild-type swarm size (Fig. 4B). Most interestingly, the swarm size of the *mcpU* deletion strain generated on histidine, lysine, or proline was comparable to the Δ9 swarm size. We infer that McpU is a major sensor for these three amino acids. On swarm plates containing different sugars, swarms produced by most strains were 85 to 100% of the size of the wild-type control. Only strains with deletions in *mcpU*, *mcpW*, *mcpX*, and *icpA* generated swarms that were 70 to 80% of the wild-type swarm size. In conclusion, none of the single receptor deletion strains, except Δ*mcpU*, showed a total loss of swarming proficiency on any of the substances assayed. However, most of the strains exhibited impaired responses toward all substances. This result suggests that (i) receptor proteins can sense more than one carbon source and (ii) one carbon source is detected by more than one receptor.

**Contributions of individual receptors to swimming speed and chemokinesis.** The swarm ring provides an indirect measure of motility and chemotaxis. Hence, as an additional assay of motility, the free-swimming speeds of cell populations were analyzed. Table 3 lists the swimming speeds of mutant and wild-type cells in both the absence and the presence of the strong chemoattractant proline. The *S. meliloti* wild-type strain (RU11/001) typically had a free-swimming speed of 37.4 μm/s, whereas the *cheA* deletion mutant (RU11/310) and a mutant lacking all nine receptor genes (RU13/149) had 8% and 10% increases in swimming speed, respectively. This result is in agreement with the behavior of *E. coli* cells lacking both of the high-abundance chemoreceptors (Tsr and Tar). These cells swim smoothly, because flagellar motors rotate exclusively counterclockwise due to the low basal activity of CheA (19, 25, 40). When either Tsr or Tar is expressed as a sole chemoreceptor, a normal rotational bias and concomitant run-tumble behavior are maintained (25, 40). In analogy, we expected only minute changes in swimming speed when individual receptors were missing. The absence of a receptor, exerting a stimulatory effect on kinase activity, is likely to cause an increase in swimming velocity. Such behavior was observed for six receptor deletion strains, with the Δ*mcpT* strain swimming 7% faster than the wild-type strain. Surprisingly, we noticed a decrease in swimming speed for three of the deletion strains, Δ*mcpU*, Δ*mcpY*, and Δ*mcpZ*. As one possible explanation for this behavior, we suggest that these three receptors inhibit the kinase activity of CheA in the absence of an attractant. The loss of *mcpU* had the most detrimental effect, reducing free-swimming speed by 9% from that of the wild-type control. Next, the chemokinesis responses of mutant strains were compared to the behavior of the wild-type control. Wild-type *S. meliloti* reacted to the addition of the attractant proline by a 7.5% increase in swimming speed. The *cheA* deletion strain and the Δ9 strain suffered severe losses of chemokinesis response. They swam only 0.5% faster upon addition of proline, which is 10% of the wild-type increase. Six of the nine single-deletion strains were diminished in their chemokinesis responses to various degrees. We observed the most pronounced decrease in chemokinesis for strains lacking *mcpY* or *icpA*, with a reduction of 66% or 55%, respectively. Interestingly, a mutant strain missing both internal receptor genes (RU13/107 [Table 1]) lost 74% of its chemokinesis proficiency. It should be noted that strains lacking *mcpZ*, *mcpU*, or *mcpX* exhibited a chemokinesis response that was increased 4% to 19% over that of the wild-type control.

How do free-swimming speed and chemokinesis correlate with the behavior of mutant strains on swarm plates? A balanced chemokinesis is most important for efficient swarming. Generally speaking, mutant strains with extremely weak or extremely strong chemokinesis, e.g., Δ*icpA* and Δ*mcpX* strains, generate smaller swarm rings than mutants with chemokinesis responses close to that of the wild-type control, e.g., Δ*mcpS* and Δ*mcpZ* strains. A decrease in free-swimming speed (Δ*mcpU*) is more devastating for swarming than an increase in speed (Δ*mcpT*).

**Contributions of individual receptors to the reaction to proline in capillary assays.** A standard for quantitative assessment of chemotaxis is the capillary tube assay established by Adler in 1973 (2). In this assay, the number of bacteria attracted into a capillary tube containing an attractant is measured. We determined concentration-response curves for the strong chemoattractant proline for the wild-type strain and 11 mutant strains. Wild-type cells responded optimally to proline at a concentration of 100 mM. Compared to that of wild-type cells, the taxis of the *cheA* deletion strain and the Δ9 strain was almost completely abolished (Fig. 5A to C). The attraction of strains lacking *mcpS*, *mcpW*, or *mcpZ* to proline was about 10 to 20% weaker than that of the wild-type control. Interestingly, maxi-

mum response was shifted to lower concentrations of proline (10 mM) for the Δ*mcpS* and Δ*mcpW* strains. A distinct reduction in the tactic response to proline was observed for strains with deletions in *mcpT*, *mcpV*, and *icpA*, with a 40 to 50% decreased sensitivity compared to the wild-type standard. Again, maximum response for the Δ*icpA* strain was shifted to 10 mM proline. The group of deletion strains with the weakest response comprises the Δ*mcpU*, Δ*mcpX*, and Δ*mcpY* strains. In this group, taxis toward proline was reduced by 65 to 75%. In conclusion, the capillary assays confirmed the significant role of McpU for proline taxis, as already observed on swarm plates. They also revealed two additional important receptors for proline sensing: McpX and McpY (Fig. 4B). The importance of McpY for proline sensing is verified by the weak chemokinesis effect (Table 3). The weak response of the *mcpX* deletion strain, however, was unexpected according to the swarm plate assays (Fig. 4B), although chemotaxis on swarm plates also involves cell growth and division, which in this case may have disguised the effect.

How do the results from the capillary assays correlate with chemokinesis? Mutant strains that swam as fast as the wild-type strain were only weakly impaired in the capillary assay (Δ*mcpS*, Δ*mcpW*, Δ*mcpZ*). However, mutants with strongly reduced chemokinetic capability (Δ*mcpY*, Δ*icpA*), mutants with extremely reduced swimming speed (Δ*mcpU*), and mutants that swam faster than the wild-type control after stimulation with proline (Δ*mcpT*, Δ*mcpX*) were strongly impaired in the capillary assay. Apparently, fast swimmers have difficulties entering the opening of the capillary. From these results, it is evident that the three different chemotaxis assays used in this study complement one another.

**Energy taxis is not a dominant determinant of chemotaxis in *S. meliloti*.** Receptors mediating the chemotactic behavior of *S. meliloti* can sense a wide range of chemical substances and even classes of substances (Fig. 4). We therefore asked the question whether energy taxis, a mechanism described for a related alpha-proteobacterium, is involved in the tactic response of *S. meliloti*. In *Azospirillum brasilense*, energy taxis is the dominant determinant of chemotaxis toward most chemoeffectors. Energy taxis is metabolism dependent, and behavioral responses are triggered by changes in the electron transport system (3). We thus compared the stimulation of chemokinesis by five different substrates with the stimulation caused by their nonmetabolizable analogs (Fig. 6). Clearly, in the cases of succinate, alanine, and proline, the chemokinesis responses of wild-type *S. meliloti* cells to the correspondent analogs, itaconic acid, α-amino-isobutyrate, and azetidine-2-carboxylate, are equal to or even stronger than those to the substrates themselves. This result confirms the findings of the 1993 study by Robinson and Bauer (55), who used itaconic acid as an attractant in capillary assays. For the two sugars tested, glucose and lactose, the responses to the analogs, 2-methylglucoside and isopropylthiogalactoside, were weaker but still significant. We therefore conclude that the responses to organic

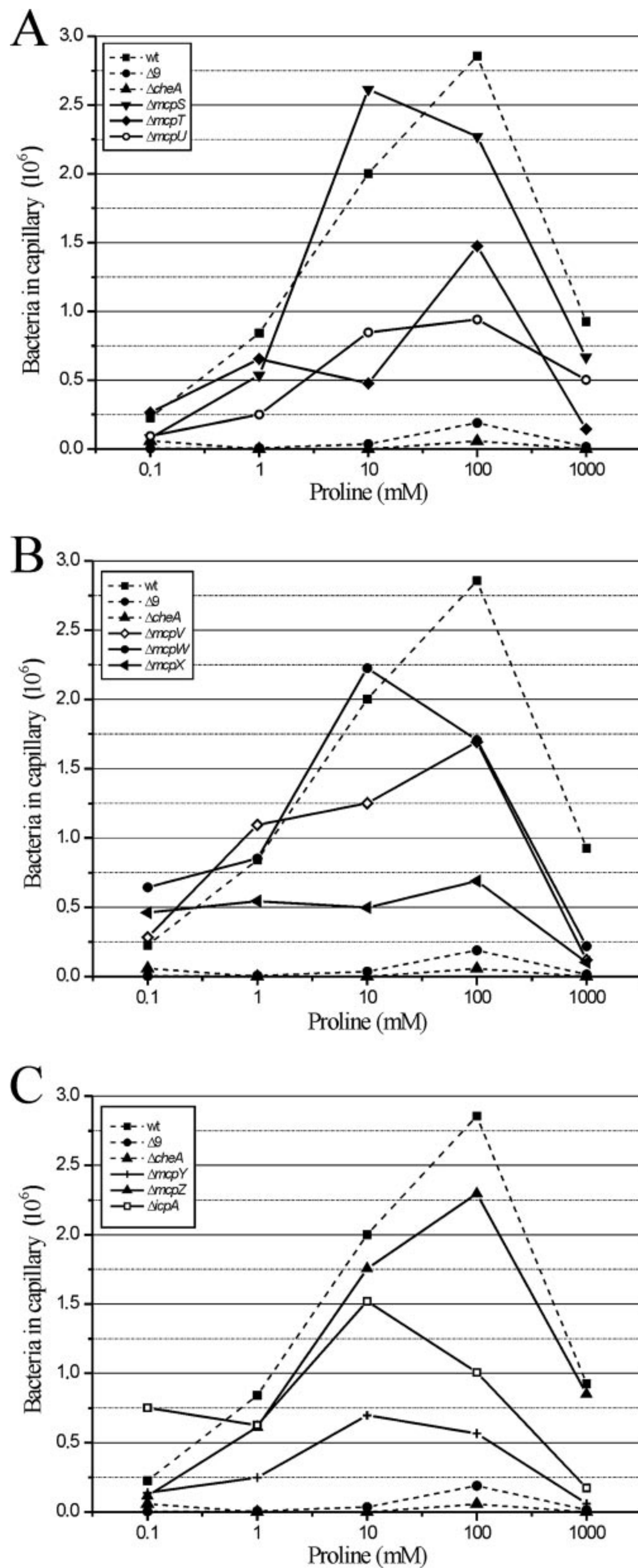


FIG. 5. Concentration-response curves for wild-type and chemotaxis mutant strains. Results for capillary assays performed with the wild-type (■), Δ9 (●), and Δ*cheA* (▲) strains, connected by dashed lines, are given as references in all graphs. Shown are curves for the Δ*mcpS* (▼), Δ*mcpT* (◆), and Δ*mcpU* (○) strains (A), for the Δ*mcpV* (◇), Δ*mcpW* (●), and Δ*mcpX* (◀) strains (B), and for the Δ*mcpY* (+), Δ*mcpZ* (▲), Δ*icpA* (□) strains (C). Each curve represents the mean of two experiments, each in triplicate, after background subtraction ($0.4 \times 10^5$ to $2.3 \times 10^5$ bacteria in capillaries with buffer).

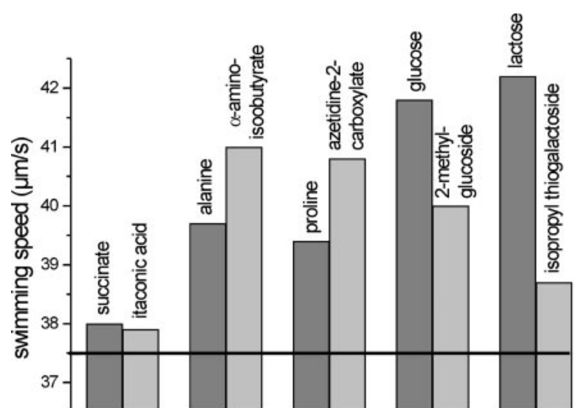


FIG. 6. Effects of substrate analogs on free-swimming speed of wild-type *S. meliloti*. Substrates were diluted from concentrated stock solutions in RB minimal medium, which was buffered with 10 mM phosphate (pH 7.0). The horizontal line marks the swimming speed of unstimulated cells. For each substrate, 1,000 individual tracks from at least five independent cell populations were analyzed by the Hobson BacTracker system.

acids and amino acids are not mediated by the mechanism of energy taxis. However, further investigations are needed to rule out this possibility for sugar sensing.

**Chemoreceptor genes are transcribed at different levels.** Transcription from chemoreceptor gene promoters was probed by using plasmid-borne *lacZ* fusions in the wild-type background. Table 4 lists the positions and lengths of the constructs relative to the start codon of each gene. β-Galactosidase activities were determined from cells grown to exponential phase in RB minimal medium layered on Bromfield agar plates. Seven of the nine reporter constructs induced significant enzyme activity, while the P*mcpV* construct was inactive and P*mcpS* (P*che2*) had only minimal activity. Nevertheless, transcription of these genes was anticipated, because deletions in their coding regions had distinct effects in our chemotaxis assays (Fig. 2, 4, and 5; Table 3). At this point, we can only speculate that the upstream region, fused to the reporter gene, was too short or that the real start codon is further downstream of the one originally predicted. Thus, the reasons for the inactivity of these constructs remain unclear. The activities of P*mcpW*, P*mcpZ*, and P*icpA* were about five times higher than those of the low-expression genes *mcpT* and *mcpY*. However, the promoters of *mcpX* and *mcpU* were clearly the strongest. Together they comprise more than 50% of the combined promoter activities, underscoring their importance for the chemotactic response of *S. meliloti*.

## DISCUSSION

The increasing number of genome sequences reveals the diversity and complexity of chemotactic signaling pathways in prokaryotes. Most motile bacterial species with available genome sequences have multiple homologs of *che* genes and more receptor genes than the five present in the well-studied enterobacterial species. For example, in the alpha subgroup of proteobacteria, *Agrobacterium tumefaciens* has 20, *C. crescentus* has 18, and *Rhodobacter sphaeroides* has 13 MCPs or transducer-like proteins. Some of these receptor proteins have no periplasmic domains, owing to the lack of obvious transmembrane regions (28, 44, 50). The motility system of the alphaproteobacterium *S. meliloti* deviates from the enterobacterial paradigm in its mode of flagellar rotation and in the ability to increase its swimming speed as a reaction toward attractants (8, 58, 64).

In this study, we analyzed the roles of seven transmembrane and two cytosolic receptor proteins in chemotaxis and chemokinesis. Each of the cytosolic receptor proteins has distinctive molecular features. McpY possesses two PAS domains, which are typically involved in sensing redox potential, oxygen, or light (71), while IcpA lacks the otherwise highly conserved methylated residues (Fig. 1). A possible role for these internal receptors will be discussed below.

Four of the MCP polypeptides have conserved C-terminal pentapeptides related to the one found for *E. coli* high-abundance receptors. For *E. coli* MCPs, this motif serves as a docking site for enzymes involved in adaptation. It remains to be experimentally investigated if the same holds true for the (N/D)WEEF pentapeptide in McpS, McpW, McpX, and McpY. Unlike the situation for *E. coli*, however, there is no correlation between high expression levels and the presence of the motif in *S. meliloti* MCPs.

We were unable to detect promoter activity for the 5′ upstream region of *mcpV*. However, two lines of evidence are in favor of expression of this gene: (i) deletion in the coding region resulted in reduced chemotactic proficiency on swarm plates (Fig. 4) and in capillary assays (Fig. 5); (ii) cells carrying a chromosomal 3′ *gfp* fusion to *mcpV* fluoresce at the cell poles (data not shown).

*S. meliloti* strains lacking all nine receptors or the histidine kinase CheA were still able to form swarm rings with 30% of the size of a swarm ring generated by the wild-type control (Fig. 2 and 4). In contrast, *E. coli* cells that fail to tumble, e.g., due to the lack of receptor proteins, are nearly incapable of moving from the inoculation point. Mutant cells that swim smoothly inevitably collide with the agar walls and get trapped

TABLE 4. In vivo *mcp* promoter activity

| Plasmid[a] (*lacZ* fusion) | Cloned region[b] (bp) | β-Galactosidase activity[c] (Miller units) |
|---|---|---|
| pRU2899 (*mcpS* [*che2*]) | −860/+9 | 4 |
| pRU2728 (*mcpT*) | −317/+3 | 29 |
| pRU2283 (*mcpU*) | −418/+38 | 235 |
| pRU2896 (*mcpV*) | −413/+2 | 0 |
| pRU2784 (*mcpW*) | −301/+2 | 127 |
| pRU2994 (*mcpX*) | −585/+5 | 417 |
| pRU2898 (*mcpY*) | −723/+63 | 29 |
| pRU2787 (*mcpZ*) | −407/+2 | 154 |
| pRU2250 (*icpA* [*che*]) | −1804/+170 | 156 |

[a] Transcription from nine chemoreceptor promoters was assessed with plasmid-borne *lacZ* fusions (Table 1) for the wild type (RU11/001) during exponential growth. Cells diluted in RB medium were layered on Bromfield agar plates and grown to an $OD_{600}$ of 0.15 to 0.25 (see Materials and Methods). The *che* operon (*che*) is composed of the *icpA*, *orf2*, *cheY1*, *cheA*, *cheW*, *cheR*, *cheB*, *cheY2*, *cheD*, and *orf10* genes (66). The second *che* operon (*che2*) is localized on the *symA* plasmid and composed of *cheR*, *cheW*, *mcpS*, *cheA*, and *cheB* (14, 26).

[b] Length of the cloned promoter region upstream/downstream of the start codon.

[c] β-Galactosidase activities (46) from three to five independent experiments were averaged.

in the agar (76). The intrinsic speed modulation in the flagellar motor of *S. meliloti* presumably enables cells to back away from obstructions in the agar. This behavior can explain the larger swarm sizes generated by mutant cells. According to the present data, the possibility that the residual chemokinesis activity of mutant strains accounts for this behavior as well cannot be excluded (Table 3).

We can only speculate what factor(s) could trigger the minor increase in swimming speed of the Δ9 or Δ*cheA* strain when stimulated by an attractant. The chromosome of *S. meliloti* contains a total of two *cheA* (one in each chemotaxis operon) and four *cheW* genes. However, participation of CheA2 in the chemotactic response is unlikely, because a *cheA1 cheA2* double-deletion strain showed the same residual chemokinesis activity (data not shown). Two additional pathways seem plausible: (i) there are other two-component regulatory systems that might target the flagellar motor either directly or by cross talk with CheA and/or CheY2; (ii) fumarate or its metabolites might directly modulate rotary speed. Fumarate has been reported as a regulator of motor switching for *E. coli* (10, 47).

In the absence of an attractant, *E. coli* MCPs stimulate the kinase activity of CheA and consequently increase the phosphorylated CheY concentration, resulting in a higher tumbling bias. The same effect is seen for *S. meliloti*; disruptions in six of the receptor genes result in increases in swimming speed to various degrees. This reaction was most pronounced for a strain lacking *mcpT*. Conspicuously, three receptor deletion strains exhibited decreases in free-swimming speed. This behavior was most distinctly observed for a strain lacking *mcpU* (Table 3). It is possible that McpU inhibits the kinase activity of CheA in the absence of an attractant, thereby causing an increase in swimming speed. The effect of McpU on kinase activity will be explored using phosphorylation assays in vitro. Besides McpU, McpX and McpY were also major players in proline sensing (Fig. 5). Interestingly enough, the periplasmic region of McpU contains a TarH domain, which is homologous to the four-helix, ligand-binding domain in *E. coli* transmembrane MCPs. The complex domain structure supports our experimental findings of McpU being a major chemoreceptor. The function of the TarH domain needs to be further elucidated, as well as that of the Cache_1 domain in McpU and McpX.

Receptors mediating the chemotactic behavior of *S. meliloti* can sense a wide range of chemical substances and even classes of substances (Fig. 4). It also becomes apparent from our study that the functions of receptors in *S. meliloti* are redundant. That is to say that more than one receptor contributes to detecting chemical stimuli and to producing an output signal that controls the speed of the flagellar motor. In conclusion, heterologous receptors all work as a team to generate a collaborative signaling behavior, ensuring the functionality and robustness of the chemotaxis system in *S. meliloti*.

It is evident that the two cytoplasmic receptors are important for the chemokinesis response of *S. meliloti* (Table 3). We speculate that IcpA and McpY are sensors for the metabolic state of the cell. Currently, we are investigating the roles of McpY and IcpA in chemokinesis to various attractants. What is the function of the PAS domains in McpY? Up to now, no data have revealed redox, aerotactic, or phototactic behavior of *S. meliloti* (78). In addition, deleting either of the two PAS domain-encoding DNA regions had no effect on the chemotactic behavior of the resulting mutant strains on swarm plates (data not shown). Thus, unlike for *E. coli* Aer (16) and *Halobacterium salinarum* HemAT (35), it is improbable that the PAS domains of McpY bind cofactors such as flavin adenine dinucleotide or heme. Further experiments are needed to determine whether McpY and IcpA are indeed localized in the cytosol or whether they are attached to the membrane. If they are membrane bound, do they colocalize with the transmembrane receptors? These and other questions will be addressed in our future studies.

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