Sinorhizobium meliloti Chemoreceptor McpU Mediates Chemotaxis toward Host Plant Exudates through Direct Proline Sensing

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Bacterial chemotaxis is an important attribute that aids in establishing symbiosis between rhizobia and their legume hosts. Plant roots and seeds exude a spectrum of molecules into the soil to attract their bacterial symbionts. The alfalfa symbiont *Sinorhizobium meliloti* possesses eight chemoreceptors to sense its environment and mediate chemotaxis toward its host. The methyl accepting chemotaxis protein McpU is one of the more abundant *S. meliloti* chemoreceptors and an important sensor for the potent attractant proline. We established a dominant role of McpU in sensing molecules exuded by alfalfa seeds. Mass spectrometry analysis determined that a single germinating seed exudes 3.72 nmol of proline, producing a millimolar concentration near the seed surface which can be detected by the chemosensory system of *S. meliloti*. Complementation analysis of the *mcpU* deletion strain verified McpU as the key proline sensor. A structure-based homology search identified tandem Cache (calcium channels and chemotaxis receptors) domains in the periplasmic region of McpU. Conserved residues Asp-155 and Asp-182 of the N-terminal Cache domain were determined to be important for proline sensing by evaluating mutant strains in capillary and swim plate assays. Differential scanning fluorimetry revealed interaction of the isolated periplasmic region of McpU (McpU₄₀₋₂₈₄) with proline and the importance of Asp-182 in this interaction. Using isothermal titration calorimetry, we determined that proline binds with a K_d (dissociation constant) of 104 μ M to McpU₄₀₋₂₈₄ while binding was abolished when Asp-182 was substituted by Glu. Our results show that McpU is mediating chemotaxis toward host plants by direct proline sensing.

Rapidly changing environmental conditions make soil a challenging environment for bacteria to persist. Motile soil bacteria use chemotaxis to navigate through the soil and to find optimal surroundings for survival. One important group of soil bacteria are symbiotic rhizobia, which fix atmospheric nitrogen to forms utilizable by its host plant. In particular, crop legumes, such as peas, soybeans, and alfalfa, form symbiotic relationships with *Rhizobium leguminosarum*, *Bradyrhizobium japonicum*, and *Sinorhizobium meliloti*, respectively (1, 2). Symbiosis partners have evolved a complex and specific molecular dialogue through the exchange of chemical signals, which direct bacterial species to the roots of host plants (3, 4). The rhizosphere is a narrow zone of soil that is influenced by root secretions, while the spermosphere is defined as the zone of soil surrounding a germinating seed. Roots and seeds alike have been shown to exude different types of compounds for the ultimate purposes of successful establishment and proliferation of beneficial microbial communities (5, 6). Host plant exudates recruit symbiotic rhizobia by inducing positive chemotaxis, which allows microbes to accumulate in the rhizosphere (7). Various attractants are exuded from the roots of the *S. meliloti* host, alfalfa (*Medicago sativa*), including sugars, amino acids, carboxylic acids, and phenolic compounds (8–12), but knowledge of substances exuded by germinating seeds is greatly lacking (13–18). Information about how *S. meliloti* perceives seed-derived attractants may aid in symbiotic efficiency, and ultimately greater crop yield by enhancing the recruitment of *S. meliloti* to the spermosphere of germinating seeds.

Chemotaxis is directed movement based on the perception of chemical stimuli. *S. meliloti* and other soil bacteria from the *Rhizobiaceae* family such as *Agrobacterium tumefaciens*, *Bradyrhizobium japonicum*, and *Rhizobium leguminosarum* use their chemotactic trait to move toward roots of their host plants, while chemotaxis-compromised strains are outcompeted by wild-type bacteria (19–23). However, little is known about the specific role

of individual chemoreceptors in this process. In *R. leguminosarum*, an *mcpC* chemoreceptor mutant is severely diminished in nodulation occupancy compared to the wild type (24). To date, there are no studies characterizing the specificity of a rhizobial chemoreceptor for host-derived signals.

Previous work has characterized important aspects of *S. meliloti* chemotaxis and how it differs from the *Escherichia coli* paradigm regarding the number and domain topology of chemoreceptors (9), the two-component regulatory system (25–27), and the unidirectional flagellar motor (28–31). *Escherichia coli* has four conventional chemoreceptors, called methyl-accepting chemotaxis proteins (MCPs) and one unorthodox receptor, Aer (32). Aer and the four MCPs share a highly conserved, C-terminal signaling domain that forms a ternary complex with two cytoplasmic chemotaxis proteins, CheA, a histidine kinase, and CheW, an adaptor protein. In the absence of an attractant, CheA is autophosphorylated and subsequently transfers the phosphoryl group to the response regulator protein, CheY (33, 34). Phosphorylated CheY interacts with the cytoplasmic face of the flagellar motor and controls the swimming paths of bacteria (35–37). Orthodox MCPs have an N-terminal periplasmic ligand-binding domain, which have been extensively studied in *E. coli*, along with each chemoreceptor's repertoire of detected chemotactic stimuli (38–42).

S. meliloti has nine genes coding for putative chemoreceptors (9, 43). We have shown that eight of these receptors participate in

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TABLE 1 Bacterial strains and plasmids

Strain or plasmid	Relevant characteristics ^a	Source or reference
Strains		
<i>E. coli</i>		
DH5α	<i>recA1 endA1</i>	78
M15/pREP4	Km ^r ; <i>lac ara gal mtlF recA uvr</i>	Qiagen
S17-1	<i>recA endA thi hsdR</i> RP4-2 Tc::Mu::Tn7; Tp ^r Sp ^r	56
<i>S. meliloti</i>		
BS182	Sm ^r ; <i>mcpU</i> with codon 182 changed from GAT to GCC (D182A)	This study
BS183	Sm ^r ; <i>mcpU</i> -EGFP gene with codon 182 changed from GAT to GCC (D182A)	This study
BS184	Sm ^r ; <i>mcpU</i> with codon 155 changed from GAC to GCC (D155A)	This study
BS185	Sm ^r ; <i>mcpU</i> -EGFP gene with codon 155 changed from GAC to GCC (D155A)	This study
BS187	Sm ^r ; <i>mcpU</i> with codon 182 changed from GAT to GAG (D182E)	This study
RU11/001	Sm ^r ; spontaneous streptomycin-resistant wild-type strain	79
RU11/828	Sm ^r ; $\Delta mcpU$	9
RU13/149	Sm ^r ; $\Delta mcpS \Delta mcpT \Delta mcpU \Delta mcpV \Delta mcpW \Delta mcpX \Delta mcpY \Delta mcpZ \Delta icpA$ ($\Delta 9$)	26
RU13/285	Sm ^r ; $\Delta mcpS \Delta mcpT \Delta mcpV \Delta mcpW \Delta mcpX \Delta mcpY \Delta mcpZ \Delta icpA$ ($\Delta 8$); <i>mcpU</i> crossed-back into RU13/149	This study
RU13/301	Sm ^r ; <i>mcpU</i> -EGFP gene	44
Plasmids		
pACYC184	Cm ^r Tc ^r ; source of <i>lacI^q</i>	80
pBBR1-MCS2	Km ^r	81
pBS189	Ap ^r ; <i>lacI^q</i> fragment from pBS189 cloned into the SacI site of pBBR1-MCS2	This study
pBS373	Ap ^r ; 735-bp SphI/HindIII PCR fragment containing <i>mcpU</i> , 118 to 852 bp (aa 40 to 284) cloned into pQE30	This study
pBS383	Ap ^r ; 735-bp SphI/HindIII PCR fragment containing <i>mcpU</i> , 118 to 852 bp (aa 40 to 284), with <i>mcpU</i> codon 155 changed from GAC to GCC (D155A) cloned into pQE30	This study
pBS384	Ap ^r ; 735-bp SphI/HindIII PCR fragment containing <i>mcpU</i> , 118 to 852 bp (aa 40 to 284), with <i>mcpU</i> codon 182 changed from GAT to GCC (D182A) cloned into pQE30	This study
pBS390	Ap ^r ; 735-bp SphI/HindIII PCR fragment containing <i>mcpU</i> , 118 to 852 bp (aa 40 to 284), with <i>mcpU</i> codon 182 changed from GAT to GAG (D182E) cloned into pQE30	This study
pBS1053	2,124-bp HindIII/XbaI PCR fragment containing <i>mcpU</i> cloned into pBS189	This study
pK18mobsacB	Km ^r ; <i>lacZ mob sacB</i>	82
pQE30	Ap ^r ; expression vector	Qiagen

^a Nomenclature is presented according to Bachmann (83) and Novick et al. (84). Cm^r, chloramphenicol; Tp^r, trimethoprim resistance; Tc^r, tetracycline resistance; Ap^r, ampicillin resistance; Sm^r, streptomycin resistance; Km^r, kanamycin resistance; aa, amino acids.

chemotaxis, while the *mcpS* gene is not expressed when cells are motile, and presumably regulates processes other than chemotaxis (9, 44). Seven of the *S. meliloti* chemoreceptors are classical MCPs, McpT to McpZ, and one receptor, IcpA, lacks the conserved residues that typically serve as methyl-accepting sites to control adaptation. Six of the MCPs are located in the cytoplasmic membrane via two membrane-spanning regions, whereas McpY and IcpA lack such hydrophobic regions (9). All chemoreceptors vary in their ligand-binding domains; however, McpU, McpV, and McpX contain specialized domains such as Cache and TarH signaling domains (9), which are known to bind small molecules such as amino acids (45, 46). McpY possesses two Per-Arnt-Sim (PAS) domains, which typically sense redox potential, oxygen, or light (47). Deletion of individual receptor genes causes differential impairments in the chemotactic response toward various sugars, amino acids, and organic acids (9). The exact function of *S. meliloti* chemoreceptors and mode of attractant binding is not known, and the identification of ligands, and in particular, plant-borne signaling molecules, is a focus of our work.

McpU is one of the most strongly expressed chemoreceptors in *S. meliloti* and is a major sensor for the potent attractant proline (9). We show here that proline is sensed through direct binding to the periplasmic region of McpU. We also demonstrate that McpU mediates positive chemotaxis to host seed exudates and quantify

the amount of proline exuded by germinating seeds. In conclusion, sensing of seed-derived proline by McpU plays a significant role in host plant recognition.

MATERIALS AND METHODS

Bacterial strains and plasmids. Derivatives of *E. coli* K-12 and *S. meliloti* MV II-1 (48) and the plasmids used are listed in Table 1.

Media and growth conditions. *E. coli* strains were grown in lysogeny broth (LB) (49) at 37°C. *S. meliloti* strains were grown in TYC (0.5% [wt/vol] tryptone, 0.3% [wt/vol] yeast extract, 0.13% CaCl₂·6H₂O [wt/vol]; pH 7.0) at 30°C (50). Motile cells for capillary assays were grown for 2 days in TYC, diluted 1:2 in 3 ml of TYC, and grown for 11 h. Cultures were then diluted 1:100 in 10 ml of RB minimal medium [6.1 mM K₂HPO₄, 3.9 mM KH₂PO₄, 1 mM MgSO₄, 1 mM (NH₄)₂SO₄, 0.1 mM CaCl₂, 0.1 mM NaCl, 0.01 mM Na₂MoO₄, 0.001 mM FeSO₄, 20 μg of biotin/liter, 100 μg of thiamine/liter (11)], layered on Bromfield agar plates (27), and incubated at 30°C for 14 h to an optical density at 600 nm (OD₆₀₀) of 0.12 ± 0.02. All strains were motile and exhibited swimming characteristics as described by Meier et al. (9). The following antibiotics were used at the indicated final concentrations: for *E. coli*, ampicillin at 100 μg/ml and kanamycin at 50 μg/ml, and for *S. meliloti*, neomycin at 120 μg/ml and streptomycin at 600 μg/ml.

Preparation of seed exudates. The *Medicago sativa* cultivar “Guardman II” (registration number CV-203, PI 639220) used in the present study was developed from the extensively studied cultivar “Iroquois” by the Cornell University Agricultural Experiment Station, New York State

College of Agriculture and Life Sciences, Cornell University, Ithaca, NY (48). Seeds (0.1 g, $\sim 47 \pm 1$ seeds) were placed in a 50-ml conical tube and washed four times with 35 ml of autoclaved, sterile-filtered water. Next, the seeds were washed once with 3 ml of 6% commercial bleach for 3 min and four times with 35 ml of autoclaved, sterile-filtered water under slow agitation. The seeds were then transferred to a 125-ml Erlenmeyer flask containing 20 ml of autoclaved, sterile-filtered water. The seeds were jostled to distribute them evenly at the bottom of the flask and germinated without shaking at 30°C for 24 h. After incubation, the germination efficiency was ca. 95% as determined by radicle emergence. To harvest the exudate, 19 ml of seed supernatant was removed from the flask, placed into a 50-ml conical tube, and flash frozen in liquid nitrogen. The frozen sample was freeze-dried for 72 h and stored at -20°C . Seed exudates were tested for bacterial contamination by microscopic examination and by plating 20 μl on TYC plates, followed by incubation at 30°C for 24 h. Contaminated samples were not included in the present study.

Quantification of proline in alfalfa seed exudates by liquid chromatography-mass spectrometry (LC-MS). Seed exudate residue from three biological replicates was thawed to room temperature, resuspended in 1 ml of 0.1% formic acid in water and sonicated for 10 min. The samples were centrifuged at $3,450 \times g$ for 10 min to remove insoluble material. Two 120- μl aliquots were removed from the supernatant of each biological replicate and transferred to separate 1.5-ml microcentrifuge tubes and centrifuged at $14,000 \times g$ to pellet remaining insoluble material. A 100- μl aliquot of supernatant was then processed by using an "EZ:faast for Free Physiological Amino Acid Analysis by LC-MS" kit according to the manufacturer's instructions (Phenomenex, Torrance, CA). Briefly, EZ:faast consists of a solid-phase extraction step, followed by a derivatization and extraction of free amino acids. The derivatized amino acids were analyzed by LC-MS using the supplied EZ:faast AAA-MS on an Agilent 1100 series HPLC (Agilent Technologies, Santa Clara, CA) coupled with an Applied Biosystems 3200 Q Trap LC-MS/MS System (AB Sciex, Framingham, MA). Calibration solutions were made separately from the Phenomenex kit with L-proline (Sigma-Aldrich, St. Louis, MO). Proline standard solutions ranging from 55 to 220 nmol/ml were derivatized alongside the exudate samples to establish the proline calibration curve. Using Analyst software (AB Sciex, Concord, Ontario, Canada) for data analysis, the peak area of the internal standard, homoarginine, was used to normalize the concentration of proline and given as average derived from three biological replicates, each processed twice, with duplicate injections.

Swim plate assay. Swim plates containing RB minimal medium complemented with 10^{-4} M L-proline, 0.27% Bacto Agar, and various concentrations of IPTG (isopropyl- β -D-thiogalactopyranoside) were inoculated with 3- μl droplets of the test culture, followed by incubation at 30°C for 3 to 4 days (9).

Traditional capillary assays. Traditional capillary assays were performed essentially as described by Adler (51), with minor modifications (9, 52). Cells were harvested by centrifugation at $3,000 \times g$ for 5 min at room temperature and suspended in RB minimal medium 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. For each capillary, 400 μl of bacterial suspension was used to make a bacterial pond. Capillary tubes (1- μl disposable micropipettes; Drummond Microcaps) were sealed at one end and filled with attractant dissolved in RB minimal medium. The capillaries were inserted open end first into the bacterial pond and incubated for 2 h at 22.5°C. Capillaries were removed, the sealed end was cut off, and the complete content was transferred into 1 ml of RB minimal medium. Dilutions were plated in duplicate on TYC plates containing streptomycin. After incubation for 3 days at 30°C, the colonies were counted.

Agarose capillary assays. Agarose capillary assays were performed essentially as described by Grimm and Harwood (53), with minor modifications. Dried seed exudate was thawed to room temperature, dissolved in acetone-water (7:3 [vol/vol]), and centrifuged at $3,000 \times g$ for 5 min. The soluble fraction was concentrated to dryness, dissolved in RB to a concen-

tration of 1.5 mg/ml, and diluted 1:10 in molten 1.1% low-melting-temperature agarose (NuSieve GTG). To fill the capillaries, 0.5- μl capillaries (Drummond Microcaps) with one end sealed were heated with a Bunsen burner, and the open end was placed into the molten mixture. The mixture was allowed to solidify, and the open end of the capillary was placed into a circular chemotaxis chamber formed by a microscope slide, a rubber O-ring with an inner diameter of 8.5 mm and a height of 1 mm (Sarstedt), and a coverslip. Cells were harvested and suspended in RB to an OD_{600} of 0.20. An 81- μl aliquot of *S. meliloti* suspension was placed in the chamber before the coverslip was in place. The open end of the capillary was observed at $\times 100$ magnification under dark phase using a Nikon Optiphot-2. Pictures were taken using a Q-Imaging Micropublisher camera.

Hydrogel capillary assays. Capillaries containing a cross-linked hydrogel instead of agarose were used because of their improved properties in preventing cells from entering the capillaries. The inner glass surfaces of 0.5- μl capillaries (Drummond Microcaps) were cleaned with a 3:1 solution of concentrated sulfuric acid, and 30% hydrogen peroxide before capillaries were treated with a 1% (vol/vol) solution of 3-(trichlorosilyl)propyl methacrylate (Sigma-Aldrich) diluted in paraffin oil at room temperature for 10 min. After a thorough rinse with 100% ethanol and removal of residual ethanol in a nitrogen gas stream, the capillaries were baked at 95°C for 10 min. A 10% (wt/vol) solution of poly(ethylene glycol) diacrylate with an average M_n of 6,000 (Sigma-Aldrich) in phosphate-buffered saline (pH 7.4) was mixed with 10% (wt/vol) Irgacure2959 (Sigma-Aldrich) in 70% ethanol at a ratio of 1:20 to form the hydrogel solution, which was subsequently pulled into the capillaries with a rubber suction bulb. After the capillaries were submerged in hydrogel solution, photopolymerization was performed for 20 s using a 365 nm, 18 W cm^{-2} OmniCure S1000 UV light source (EXFO Photonic Solutions, Inc., Vanier, Quebec, Canada). Hydrogel capillaries were soaked in distilled H_2O for 5 h with five water exchanges and equilibrated with L-proline solutions using the same method. One end of each capillary was sealed with machine grease (Apiezon, Manchester, United Kingdom), and chemotaxis assays were carried out as described for the agarose capillary assays.

DNA methods and genetic manipulations. *S. meliloti* DNA was isolated and purified as described previously (27). Plasmid DNA was purified with a Wizard Plus SV Miniprep system (Promega). DNA fragments or PCR products were purified from agarose gels using a Wizard SV gel and PCR clean-up system (Promega). PCR amplification of chromosomal DNA was carried out according to published protocols (54). Deletion and codon replacement constructs were created by PCR and overlap extension PCR as described by Higuchi (55). These constructs were cloned into the suicide vector pK18mobsacB, which was then used to transform *E. coli* S17-1. The plasmid was conjugally transferred to *S. meliloti* by filter mating according to the method of Simon et al. (56). Allelic replacement was achieved by sequential selections on neomycin and 10% (wt/vol) sucrose as described previously (27). Confirmation of allelic replacement and elimination of the vector was obtained by gene-specific primer PCR and DNA sequencing.

Isolation of *S. meliloti* cell membranes. *S. meliloti* strains BS183, BS185, RU11/001, and RU13/301 (Table 1) were grown in *Sinorhizobium* motility medium (RB minimal medium, 0.2% mannitol, 2% TY) (11, 57) to an OD_{600} of 0.26 ± 0.10 . A portion (250 ml) of cell culture normalized to an OD_{600} of 0.26 was harvested by centrifugation at $5,000 \times g$ for 5 min at 4°C. The cells were suspended in 20 ml of 1 mM phenylmethylsulfonyl fluoride (PMSF)–20 mM Tris-HCl (pH 8.0) plus 5 μg of DNase I/ml. After two passages through a French press at 20,000 lb/in², the resulting extract was freed of unbroken cells by centrifugation at $2,000 \times g$ for 2 min. Broken cells were centrifuged at $200,000 \times g$ for 60 min at 4°C, yielding the pellet as the membrane fraction. Membrane fractions were resuspended in 3.5 ml of 1 mM PMSF–20 mM Tris-HCl (pH 8.0) and homogenized for use in immunoblots. Prior to sodium dodecyl sulfate (SDS)-gel electrophoresis,

40- μ l portions of the samples were mixed with 25 μ l of SDS sample buffer containing 0.5% β -mercaptoethanol. Prior to SDS-electrophoresis, the samples were heated to 100°C for 10 min.

Immunoblotting. Homogenized membrane fractions were separated in 10% acrylamide gels, transferred to Trans-Blot nitrocellulose (Bio-Rad Laboratories, Hercules, CA), and probed using monoclonal antibodies raised against green fluorescent protein (GFP; Living Colors GFP monoclonal antibody; Clontech, Mountain View, CA) at a 1:20,000 dilution. Blots were incubated with sheep anti-mouse horseradish peroxidase-linked whole immunoglobulin (GE Healthcare Life Sciences, Pittsburgh, PA) diluted 1:40,000. Detection was achieved by enhanced chemiluminescence using SuperSignal West Pico chemiluminescent substrate (Thermo Scientific, Rockford, IL) using Hyperfilm ECL (Amersham, Pittsburgh, PA). Films were scanned by using an Epson Perfection 1640SU and Corel Photo-Paint 10 software. Analysis of the scans was performed using ImageJ and Origin 8.1 software.

Expression and purification of McpU-PR. The recombinant ligand-binding, periplasmic region of McpU (McpU-PR; McpU₄₀₋₂₈₄) and its single amino acid substitution variants were overproduced from plasmid pBS373, pBS383, pBS384, and pBS390 in *E. coli* M15/pREP4 (Table 1). Three liters of cell culture were grown to an OD₆₀₀ of 0.8 at 37°C in LB containing 100 μ g of ampicillin/ml and 50 μ g of kanamycin/ml, and gene expression was induced by 0.6 mM IPTG. Cultivation was continued for 4 h at 25°C until harvest. Cells were suspended in 50 ml of column buffer (500 mM NaCl, 20 mM imidazole, 1 mM PMSF, 20 mM NaPO₄ [pH 7.0]) and lysed by three passages through a French pressure cell at 20,000 lb/in² (SLM Aminco, Silver Spring, MD), and the soluble fraction was loaded onto a 5-ml NTA column (GE Healthcare Life Sciences) charged with Ni²⁺. Protein was eluted from the column with elution buffer (500 mM NaCl, 500 mM imidazole, 1 mM PMSF, 20 mM NaPO₄ [pH 7.0]). Pooled fractions were concentrated by ultrafiltration on regenerated cellulose membranes (10-kDa cutoff) and further purified by Äktaprima Plus gel filtration HiPrep 26/60 Sephacryl S-300 HR (GE Healthcare Life Sciences). The column was equilibrated and developed in 100 mM NaCl–50 mM HEPES (pH 7.0) at 0.5 ml/min, and protein-containing fractions were combined and stored at 4°C.

Thermal denaturation studies. Differential scanning fluorimetry (DSF) experiments were performed using an ABI 7300 real-time PCR system (Applied Biosystems, Foster City, CA). L-Proline was dissolved in 20 mM imidazole–500 mM NaCl–20 mM NaPO₄ (pH 6.4) and used at final concentrations of 100 μ M, 1 mM, and 10 mM. McpU-PR (the wild type and its variants) and SYPRO Orange (Invitrogen, Grand Island, NY) were diluted in the same buffer to final concentrations of 10 μ M and a final SYPRO Orange concentration of 0.7 $\times$ (from a 5,000 $\times$ stock). Experiments with 30- μ l reaction mixtures for all conditions were performed in duplicate. A temperature gradient was applied from 10 to 85°C with a 30-s equilibration at each half degree centigrade. Fluorescence was quantified using the preset FRET parameters (excitation, 490 nm; emission, 530 nm) and normalized to the lowest and highest intensities for each data set. Melting temperatures were determined by data analysis with XLfit from IDBusiness Solutions (Guildford, United Kingdom).

Isothermal titration calorimetry (ITC). McpU-PR and McpU-PR/D182E in 100 mM NaCl–50 mM HEPES (pH 7.0) were concentrated to 812 μ M using a 10-kDa regenerated cellulose membrane in a 50-ml Amicon filter unit and a 10-kDa Centricon centrifugal filter device (Millipore, Bedford, MA). Measurements were made on a VP-ITC microcalorimeter (MicroCal, Northampton, MA) at 22°C. The protein was placed in the sample cell and titrated with 10- μ l injections of 9.8 mM L-proline that had been dissolved in the flowthrough of the protein filtration step. The flowthrough fraction was titrated with the same concentration of L-proline to produce a baseline that was subtracted from the protein titration. The data analysis was carried out using the “one binding site” model of the MicroCal version of Origin 8.1 software (OriginLab, Northampton, MA).

FIG 1 Chemotactic responses of *S. meliloti* wild type (WT, RU11/001), *mcpU* deletion (Δ *mcpU*, RU11/828) and *che* strain (RU13/149, Δ *mcpS* Δ *mcpT* Δ *mcpU* Δ *mcpV* Δ *mcpW* Δ *mcpX* Δ *mcpY* Δ *mcpZ* Δ *icpA* [Δ 9]) toward alfalfa seed exudate. Strains from the early log phase in RB were tested with agarose capillaries containing 0.15 mg of alfalfa seed exudate/ml in RB solidified with 1% agarose. Photographs were taken at $\times$ 100 magnification under dark-field microscopy after 10 min for the wild type and after 20 min for the *mcpU* and *che* deletion strains, respectively.

RESULTS

McpU mediates *S. meliloti* chemotaxis toward seed exudate of its host *Medicago sativa*. Chemotaxis of rhizobacteria in the soil toward host plant roots is important in establishing symbiosis (58, 59). Since McpU is a major receptor in *S. meliloti* chemotaxis (9, 44), we analyzed the reaction of the wild type (WT; RU11/001), a strain lacking *mcpU* (Δ *mcpU*; RU11/828), and a *che* strain (Δ *mcpS* Δ *mcpT* Δ *mcpU* Δ *mcpV* Δ *mcpW* Δ *mcpX* Δ *mcpY* Δ *mcpZ* Δ *icpA* [Δ 9]; RU13/149) toward exudates of *Medicago sativa* (alfalfa) seeds. Exudates were prepared from surface-sterilized germinating seeds and tested with motile cell populations in qualitative agarose capillary assays. The wild-type strain revealed a strong positive response toward seed exudate as detected by the accumulation of cells at the mouth of the capillary (Fig. 1, top), while a

strain lacking all nine chemoreceptors genes (RU13/149) showed a complete lack of cell accumulation and is therefore defined as chemotaxis negative (*che*; Fig. 1, middle). In comparison, the *mcpU* deletion strain exhibited a very weak, but still visible response, observed as the faint accumulation of cells at the capillary opening (Fig. 1, bottom). The strongly diminished response of the $\Delta mcpU$ strain indicates that McpU is a principal chemoreceptor for attractant components in alfalfa seed exudates.

The potent chemoattractant proline is exuded by germinating alfalfa seeds. Since capillary assays revealed that McpU is an important chemoreceptor for host seed exudates and previous studies recognized McpU as a major proline sensor (9), we analyzed the amount of proline exuded by germinating alfalfa seeds. Three biological replicates of surface-sterilized seeds were germinated, and exudates were harvested and processed with the Phenomenex EZ:fast kit. The data analysis after LC-MS revealed that $1.75 \pm 0.24 \mu\text{mol}$ of proline are exuded by 1 g of seeds (201 $\mu\text{g/g}$ of seed), which equals an amount of 3.72 nmol of proline per alfalfa seed. With an average seed volume of 2.17 μl , the concentration of exuded proline at the seed surface is predicted to be 1.71 mM, which would then be allowed to diffuse from the seed into the spermosphere. Millimolar concentrations of proline have been reported to elicit an attractant response from *S. meliloti* (9, 11). In conclusion, proline is secreted by germinating alfalfa seeds in concentrations detectable by the chemotaxis system of *S. meliloti*.

McpU mediates chemotaxis of *S. meliloti* toward proline. In a previous study, we analyzed the chemotaxis behavior of single chemoreceptor deletion strains and identified McpU as a major receptor for the strong attractant proline (9). To further assess the importance of McpU for the attraction of *S. meliloti* to proline, we constructed the broad-host-range plasmid pBS1053, which allows controlled expression of *mcpU* from an inducible *lac* promoter by the addition of various concentrations of IPTG. We first performed a quantitative swim plate assay with 10^{-4} M proline (L-proline was used in all studies) as the sole carbon source. After 5 days of incubation at 30°C, the wild type formed a swim ring with a diameter of 80 mm, while the swim ring produced by the *mcpU* deletion strain was 47% of the wild-type swim ring (Fig. 2A). In comparison, a *che* strain formed a ring on proline swim plates, which had a diameter of 27% of the wild-type swim ring size (9). In the absence of the inducer IPTG, the complemented strain ($\Delta mcpU/mcpU$) formed a ring larger than that of the deletion strain (64% of the wild type). The observed partial complementation demonstrates that McpU is already expressed under these conditions, which can be explained by basal leakiness of the *E. coli lac* promoter/LacI^q repressor system. The swim ring size of the complemented strain increased with increasing IPTG concentration, reaching a plateau between 5 and 500 μM IPTG with a maximum of 91% of the wild-type swim ring at 500 μM IPTG (Fig. 2). Higher concentrations of IPTG had a marginally inhibitory effect on swim ring size (data not shown). To serve as a second assessment, we quantified the response of *S. meliloti* strains to proline in a traditional capillary assay. This test is considered the gold standard of chemotaxis assays because of its ability to quantify cell numbers in a capillary, thus allowing for accurate classification of tested chemicals. When the response of wild-type cells to 10 mM proline was tested, 1.1×10^6 cells accumulated in the 1- μl capillary after 2 h of incubation at room temperature. The *che* strain exhibited a negligible response compared to the wild type, while

FIG 2 Complementation of the chemotactic response of the *S. meliloti mcpU* deletion strain to proline. (A) Quantitative proline swim plate assay with increasing amounts of IPTG. Strains RU11/828 ($\blacktriangle$) and RU11/828 with pBS1053 ($\bullet$) were pipetted onto RB plates containing 10^{-4} M proline with various concentrations of IPTG to induce expression of *mcpU* under the control of the *lac* promoter from plasmid pBS1053. The percentages of the wild-type swim diameter on 0.27% agar are the means of three replicates, each performed in duplicate. Error bars represent the standard deviations from the mean. (B) Quantitative capillary assay of the *mcpU* deletion strain ($\Delta mcpU$, RU11/828), the complemented strain ($\Delta mcpU/mcpU$, RU11/828 with pBS1053) induced with 500 μM IPTG, and the chemotaxis-negative strain (*che*, RU13/149) with 10 mM proline. The results for each strain are the means of three experiments, each in triplicate. The means of each strain were normalized to the number of wild-type cells per capillary. Error bars represent the standard deviations from the mean.

the response of the *mcpU* deletion strain was 38% of the wild-type response (Fig. 2B). When the complemented strain was grown in the presence of 500 μM IPTG, which caused optimal recovery of proline sensing on swim plates (Fig. 2A), it attained 86% of the wild-type response (Fig. 3B). Taken together, both assays provided consistent results showing that the response of *S. meliloti* to

FIG 3 Identification of McpU residues involved in proline sensing using the structure of the *Vibrio cholerae* McpN as a model. The structure of the periplasmic amino-terminal domain of the McpN dimer (Protein Data Bank code 3C8C) is oriented to display the tandem Cache domains of *V. cholerae* McpN. The view on the right provides a close up of the alanine ligand in the binding pocket of the N-terminal Cache domain on the monomer on the right. Two aspartate residues (Asp172 and Asp201) likely coordinate the ligand. The corresponding residues in *S. meliloti* McpU, Asp155 and Asp182, were chosen for further studies.

proline is greatly dependent on McpU. Furthermore, proline taxis can be restored by the expression of *mcpU* *in trans*.

A structure-based homology search identifies the ligand-binding site in McpU. The periplasmic region of McpU contains a conserved Cache (calcium channels and chemotaxis receptors; [45]) signaling domain (amino acid residues 149 to 226; [60, 61]) and a less well-conserved TarH domain (residues 106 to 144; [61]). Members of the Cache family are widespread in both prokaryotic and eukaryotic organisms and are predicted to bind small molecules, including amino acids (45). A homologue search in the RCSB Protein Data Bank revealed that the periplasmic region of McpU (McpU-PR, residues 40 to 284) shares the greatest sequence identity (26%) with the sensor domain of McpN from *Vibrio cholerae*. The structure of the McpN sensor domain (residues 61 to 300, Protein Data Bank code 3C8C) appears as a homodimer (Fig. 3). Each polypeptide chain has an N-proximal α -helix that loops over into two consecutive Cache domains. Zooming in on either of the N-proximal Cache domains (close up in Fig. 3) reveals the ligand alanine in the binding pocket formed by the domains. The carboxyl groups of two aspartate residues, Asp-172 and Asp-201, coordinate the ligand via hydrogen bonds between the oxygen atoms and the amino group of the ligand. Alignment of McpU-PR and McpN-PR revealed a 34% identity in the region of the McpN tandem Cache domains, whereas the regions outside the tandem cache domains have only 16% identity (62), corroborating the presence of tandem Cache domains in McpU-PR. The amino acid sequence of the McpU periplasmic region was submitted for three dimensional-structure-based homology modeling in the SWISS-MODEL Workspace (63). A model was constructed with McpU-PR residues 7 to 245 based on the McpN template (3C8C) of *V. cholerae*. When the homology model of McpU-PR was superimposed with the structure of the McpN sensor domain, two aspartate residues, namely, Asp-155 and Asp-182, were conserved in the same positions as the two ligand-coordinating residues in McpN. This finding allows the prediction that the ligand binding pocket of *V. cholerae* McpN and *S. meliloti* McpU is conserved and that McpU coordinates proline in a similar manner that McpN uses to bind the ligand alanine.

Single-point mutations in the ligand-binding pocket of McpU cause a diminished chemotaxis response to proline. Ho-

mology modeling predicted two conserved aspartate residues in McpU-PR to be involved in ligand sensing. To elucidate the role of these residues in chemotaxis toward proline, we created *S. meliloti* mutants carrying single point mutations in position 155 and 182. Initially, we made alanine substitutions and analyzed the behavior of the resulting mutants in comparison to appropriate control strains in hydrogel capillary assays. The hydrogel capillary assay is an improved variation of the agarose capillary assay used earlier (Fig. 1). Wild-type cells (RU11/001) strongly accumulated around the mouth of the capillary filled with 1 mM proline (Fig. 4A), whereas the *che* (RU13/149) and the *mcpU* deletion (RU11/828) strains displayed no or a strongly reduced response to proline, respectively (Fig. 4B and C). Strain BS184 (McpU^{D155A}) exhibited a weaker response compared to the wild type, yet considerably stronger than that of the *mcpU* deletion strain (RU11/828) (Fig. 4D). In contrast, the response of strain BS182 (McpU^{D182A}) to proline was almost abolished (Fig. 4E). Therefore, we introduced a glutamate residue in position 182, which we predicted to be a less detrimental substitution. The resulting strain, BS187 (McpU^{D182E}) exhibited an intermediary response (Fig. 4F), which was weaker than the wild type but stronger than the *mcpU* deletion strain. To quantify the importance of aspartate residues 155 and 182 for proline taxis, we performed proline swim plate assays with the mutant strains and compared their behavior to the wild type (Fig. 5). All mutant strains exhibited a diminished response to proline, with the order of response ranking as $\Delta mcpU < McpU^{D182A} < McpU^{D182E} < McpU^{D155A} < \text{wild type}$, correlating with the results obtained from the hydrogel capillary assays.

To verify that the altered chemotactic responses of the mutant strains are not caused by reduced levels of mutant McpU protein, we assessed the expression of McpU variant proteins in *S. meliloti* using immunoblots. Since antibodies directly targeting McpU were not available, we created mutants strains with corresponding variants of McpU-enhanced green fluorescent protein (EGFP) in its native chromosomal locus for McpU^{D155A} and McpU^{D182A} (Table 1) and compared their expression and incorporation in *S. meliloti* cell membranes with that of McpU-EGFP (44) using a commercially available monoclonal anti-GFP antibody. Full-length McpU-EGFP fusion variant proteins (101 kDa) were present in isolated membranes at similar levels compared to wild-type

FIG 4 Chemotactic responses of *S. meliloti mcpU* mutant strains toward proline in hydrogel capillaries. (A) Wild type (RU11/001); (B) *che* deletion strain (RU13/149); (C) $\Delta mcpU$ strain (RU11/828); (D) McpU^{D155A} strain (BS184); (E) McpU^{D182A} strain (BS182); (F) McpU^{D182E} strain (BS187). Strains from early log phase in RB were tested with hydrogel capillaries containing 1 mM proline. Photographs were taken at $\times 100$ magnification under dark-field microscopy after 20 min.

McpU-EGFP (Fig. 6). Band intensities from three independent blots quantified as 114 and 91% for McpU^{D155A}-EGFP and McpU^{D182A}-EGFP, respectively, compared to McpU-EGFP. Since there is no correlation between the attractant response of the mutant strains (Fig. 4) and the respective band intensities of variant protein (Fig. 6), we concluded that both aspartate residues in McpU-PR are involved in proline sensing, with Asp-182 being more important than Asp-155.

Proline interacts with the periplasmic region of McpU involving Asp-182. To test the interaction of proline with the isolated sensing domain *in vitro*, we overexpressed and purified McpU-PR and its single amino acid variants McpU-PR^{D182A} and McpU^{D182E} fused N-terminally to a His₆ tag using affinity and size

FIG 5 Chemotactic responses of *S. meliloti mcpU* mutant strains toward proline in a quantitative swim plate assay compared to the wild-type strain. The *mcpU* deletion strain ($\Delta mcpU$, RU11/828), McpU^{D155A} (D155A, BS184), McpU^{D182A} (D182A, BS182), and McpU^{D182E} (D182E, BS187) strains were pipetted onto RB plates containing 10^{-4} M proline. The percentages of the wild-type swim diameter on 0.27% agar are the means of three replicates, each performed in duplicate. Error bars represent the standard deviations from the mean.

exclusion chromatography. Next, we monitored the thermal unfolding of isolated proteins in the presence of the fluorescent dye SYPRO orange, which binds to the hydrophobic parts of proteins as they unfold. This technique, named differential scanning fluorimetry (DSF), enables the identification of ligands that bind and stabilize purified proteins (64). Characteristically, the transition midpoint, T_m , shifts to higher temperatures upon binding of a low-molecular weight ligand. We determined the T_m of McpU-PR as 37°C, which shifted upon addition of 10 mM proline to 52°C (Fig. 7). The drastic T_m increase of McpU-PR in the presence of proline indicates that it stabilizes the protein through direct interaction. Furthermore, we observed a promotion of McpU-PR stability at lower concentrations of proline (1.0 and 0.1 mM; data not shown). When we tested the protein variants, we found that McpU-PR^{D182A} has a T_m of 29°C, which is 8°C lower than that of McpU-PR, indicative of reduced protein stability. In the presence of proline, no change in T_m was observed, demonstrating lack of interaction. For McpU-PR^{D182E}, the T_m in the absence of proline was 34°C, which only marginally increased by 2 to 36°C. Although the general stability of McpU-PR^{D182E} appears to be comparable to the wild-type protein, the small increase of T_m in the presence of proline infers a relatively weak interaction. Altogether, proline directly interacts with the sensing domain of McpU, while mutations in the binding pocket that affect ligand coordination reduce the affinity between receptor and proline.

Proline binds directly to McpU-PR. Next, we used isothermal titration calorimetry (ITC) to quantitatively assess binding of pro-

FIG 6 Immunoblot analysis of McpU and McpU-variant EGFP fusions in *S. meliloti* membrane fractions. *S. meliloti* cells were fractionated, and equal volumes of membrane fractions were electrophoretically separated, blotted onto nitrocellulose, and detected with anti-GFP monoclonal antibody. Lane 1, BS183 (McpU^{D182A}-EGFP); lane 2, RU13/301 (McpU-EGFP); lane 3, BS185 (McpU^{D155A}-EGFP); lane 4, RU11/001 (WT).

FIG 7 DSF profiles for binding of proline to McpU-PR and McpU-PR variants. Protein stability was monitored as a function of fluorescence intensity. Proteins were tested at a concentration of 10 μ M with or without 10 mM proline. McpU-PR (black squares), McpU-PR with proline (gray squares), McpU-PR^{D182A} (black circles), McpU-PR^{D182A} with proline (gray circles), McpU-PR^{D182E} (black triangles), and McpU-PR^{D182E} with proline (gray triangles).

line to the sensing domain of McpU and its binding site variant (McpU-PR^{D182E}). As shown in Fig. 8A, titration of McpU-PR with proline resulted in large exothermic heat signals that dissipate into heats of dilution. The affinity derived from the binding curve yielded a K_d (dissociation constant) of 104 μ M, a value well within

the range of reported bacterial chemoreceptor-ligand interactions (65–67). Titration of the McpU-PR^{D182E} variant caused very small exothermic heat signals and earlier saturation of heat release (Fig. 8B). Due to the extremely weak interaction, a K_d was not derived. The ITC results confirm direct binding of proline to McpU and the importance of Asp-182 for ligand binding.

DISCUSSION

The symbiotic soil bacterium *S. meliloti* uses chemotaxis to optimize movements toward nutrients and host plant-secreted attractants (9–13). This early step in host interaction allows bacteria to effectively locate infection sites along emerging roots and therefore more effectively compete for nodulation (19, 58). Alfalfa roots release a spectrum of phytochemicals in the soil, including carbohydrates, amino acids, organic acids, fatty acids, sterols, growth factors, vitamins, and flavonoids, to initiate and modulate the dialogue with its microbial symbiont (14, 68, 69). Similarly, germinating seeds exude many organic compounds, and we have shown that alfalfa seed exudates elicit a prevailing chemotactic response from *S. meliloti* (Fig. 1). An early recruitment of the microbial symbiont to the growing root, e.g., during seed germination, appears to be a plausible strategy to maximize interaction. Although flavonoids have been suggested as host-specific attractants, they elicit only a weak chemotactic response (8, 59) and likely have a short diffusion range in aqueous soil due to their hydrophobic nature. Therefore, amino acids, organic acids, and sugars are more attractive candidates to function as recruiting agents, because they are hydrophilic, and many of these substances have been shown to serve as chemoattractants for *S. meliloti* (9, 11, 12).

FIG 8 ITC of McpU-PR and McpU-PR^{D182E} with proline. Titration of McpU-PR (A) and McpU-PR^{D182E} (B) was carried out at a protein concentration of 812 μ M with 10- μ l injections of 9.8 mM proline. Reference data were produced by titrating buffer with proline and subtracting the resulting heat of ligand dilution from the experimental curves shown. Upper panels show the raw titration data, and lower panels show the normalized and dilution corrected peak areas of the raw titration data. The data were fitted with the One-Set-of-Sites model of the MicroCal version of Origin7.

In the present study, we rationally related proline exudation by germinating alfalfa seeds with *S. meliloti* chemotaxis toward proline. We found that proline is exuded from germinating alfalfa seeds in millimolar concentrations. It is known that plants exude most proteinogenic amino acids into the soil, where they can serve as biological sources of carbon and nitrogen for soil bacteria (70). In addition, certain amino acids, including proline and glutamate, function as osmoprotectants and are accumulated by a variety of bacteria during osmotic stress. This ability is particularly valuable in environments that are prone to significant variation in solute concentration, such as the rhizosphere (70, 71). Therefore, it is beneficial for microbes to seek higher concentrations of proline in the soil, and proline taxis has been reported for several soil bacteria, including *Agrobacterium* sp. strain H13-3, *Bacillus subtilis*, *Pseudomonas fluorescens*, and *S. meliloti* (11, 65, 72). Our current work and other studies presented that *S. meliloti* can sense and migrate toward proline in the micromolar to millimolar range (Fig. 2 and 4) (9, 11). Furthermore, preliminary analyses revealed a greater attraction of *S. meliloti* to host compared to nonhost legume seed exudates (data not shown). It would be interesting to see whether the capacity of host plants to exude amino acids such as proline has coevolved with the capability of bacterial symbionts to chemotactically react to these substances, thereby increasing symbiotic effectiveness. Studies are on the way to comparatively examine the secretomes of host and nonhost legumes.

Based on previous results, we focused our studies on McpU, one of eight chemoreceptors mediating *S. meliloti* chemotaxis. McpU is one of the more abundant chemoreceptors in *S. meliloti* (44), and capillary and swim plate assays of single deletion mutants identified McpU as major receptor for proline (9). We first investigated the reaction of an in-frame *mcpU* deletion strain toward exudates harvested from germinating alfalfa seeds and established that its chemotaxis response is strongly diminished (Fig. 1). Since proline is exuded in millimolar concentrations by germinating alfalfa seeds and proline elicits a strong chemotactic response of *S. meliloti* wild type but not the *mcpU* deletion strain (Fig. 2 and 4), we concluded that McpU is mediating chemotaxis toward host plants through proline sensing. However, proline is likely not the sole chemoattractant. The function of individual *S. meliloti* chemoreceptors for root colonization or nodulation has not been investigated. It will be interesting to see whether the *mcpU* deletion strain is impaired in its ability to induce nodule formation. It is worth mentioning that proline chemotaxis is not completely abolished in the $\Delta mcpU$ strain, which suggests an overlap in specificity by other chemoreceptors. It has been reported that at least three chemoreceptors in *Pseudomonas putida* have overlapping specificity for organic acids (14). In fact, *S. meliloti* McpX and McpY have been identified previously through capillary assays to contribute to proline taxis (9). McpX and McpU both possess Cache domains, which are known to bind small ligands such as amino acids (45), whereas McpY is a cytosolic protein with dual PAS (73) domains (9). Since detailed information about the proline-sensing characteristics of McpX and McpY is lacking, we can only speculate about their involvement in the recognition of plant-derived proline. Proline binding and/or cooperative signaling of receptor homodimers forming mixed trimers-of-dimers are among the possible explanations for the behavior of *mcpX* and *mcpY* receptor mutants. Behavioral studies of *S. meliloti* are hampered by the circumstance that chemoreceptor function appears to depend on the presence of a receptor ensemble (9). A strain

bearing only a chromosomal copy of *mcpU* but lacking all other receptor genes (RU13/285) behaves like a *che* strain on swim plates and in the agarose capillary assay. In addition, overexpression of *mcpU* from pBS1053 in a strain lacking all chemoreceptors (RU13/149) failed to restore proline taxis (data not shown). One plausible hypothesis for this behavior is a reduced capability of an individual chemoreceptor to form a signaling cluster (44). This rules out the possibility of analyzing the sensing range of single chemoreceptor species *in vivo* without the influence of other chemoreceptors.

Bacterial chemoreceptors can bind attractants directly to their periplasmic domains or indirectly through periplasmic substrate-binding proteins (74). Our *in vitro* binding studies revealed that McpU binds proline directly to its periplasmic region (Fig. 7 and 8). Using ITC we determined the K_d to be 104 μ M, which is within the range of K_d values obtained for proline binding to the periplasmic regions of McpC of *B. subtilis* and McpX of *V. cholerae* with 14 and 75 μ M, respectively (65, 66). It is worth mentioning that McpU exhibited exothermic binding, similar to McpX, while McpC displayed endothermic binding. The structures of their periplasmic domains were modeled after *V. cholerae* McpN and proposed to have tandem Cache domains. Interestingly, McpC and McpX were shown to bind multiple amino acid ligands (65, 66). It remains to be seen whether similar binding characteristics hold true for McpU and to what extent these putative ligands are exuded by the alfalfa host.

Since no structural data are available for McpU, we used the crystal structure of *V. cholerae* McpN complexed with alanine to model the ligand binding pocket and identify residues that coordinate proline (Fig. 3). Two conserved residues, Asp-155 and Asp-182 (Asp-172 and Asp-201 in McpN), in the N-terminal Cache domain were predicted to bind the ligand proline and were chosen for further phenotypic analyses. The moderate decrease in proline taxis caused by a mutation of Asp-155 to Ala indicates the involvement of this residue in proline binding. However, the effect is not as detrimental as an Asp-to-Ala mutation in position 182, which abolished proline taxis mediated by McpU and *in vitro* binding of proline to McpU-PR^{D182A} (Fig. 4, 5, and 7). We hypothesize that a change in the side chain of residue 155 can be relatively promiscuous, while the nature of the residue in position 182 is critical for proper proline binding. This conclusion is supported by the less impaired phenotype of a strain carrying an Asp-to-Glu mutation in position 182, which conserves the carboxyl group of the side chain (Fig. 4 and 5). The carboxyl moiety of the glutamate residue likely allows coordination of the ligand via hydrogen bonds to the amino group of the ligand, and yet the increased length of the side chain possibly reduces the size of the binding pocket, thereby weakening ligand affinity. Reduced affinity of proline to McpU-PR^{D182E} was confirmed by *in vitro* DSF and ITC binding analyses (Fig. 7 and 8). Additional information delivered by DSF was the moderate decrease in stability of the proteins with a variation in position 182. We found DSF to be a useful technique for monitoring interactions with proline. Although this technique does not provide K_d values, it can be beneficial as a first screen of mutant proteins or potential ligand interactions.

Chemoreceptor proteins vary in their genomic abundance, sequence, and domain topologies throughout the bacterial kingdom (67, 75). The number of MCPs in bacteria that establish pathogenic or symbiotic interactions with plant roots is typically high, e.g., 20 in *Agrobacterium tumefaciens* (76) and 36 in *Bradyrhizo-*

bium japonicum (77). In this view, *S. meliloti* is an ideal model organism to study chemoreception of phytochemicals, because it only has eight receptors directing chemotaxis (9, 43). Four of these receptors have unannotated ligand-binding domains, while the remaining four contain either Cache or PAS domains (9). We showed that the Cache domain in McpU directly binds proline. McpU is the first chemoreceptor in *S. meliloti* or other rhizobial bacteria for which a host plant-derived ligand was identified. Furthermore, we presented the importance of proline sensing for the recognition of the *S. meliloti* host alfalfa. Lastly, we drew the conclusion that McpU has a major contribution in host recognition. It will be interesting to decipher the role of the remaining seven chemoreceptor proteins in host recognition, as we continue the search for, possibly unique, host plant-specific attractants.

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