Molecular Microbiology (2017) 103(2), 333–346 ■
doi:10.1111/mmi.13561
First published online 11 November 2016

Sinorhizobium meliloti chemotaxis to quaternary ammonium compounds is mediated by the chemoreceptor McpX

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Summary

The bacterium *Sinorhizobium meliloti* is attracted to seed exudates of its host plant alfalfa (*Medicago sativa*). Since quaternary ammonium compounds (QACs) are exuded by germinating seeds, we assayed chemotaxis of *S. meliloti* towards betonicine, choline, glycine betaine, stachydrine and trigonelline. The wild type displayed a positive response to all QACs. Using LC–MS, we determined that each germinating alfalfa seed exuded QACs in the nanogram range. Compared to the closely related nonhost species, spotted medic (*Medicago arabica*), unique profiles were released. Further assessments of single chemoreceptor deletion strains revealed that an *mcpX* deletion strain displayed little to no response to these compounds. Differential scanning fluorimetry showed interaction of the isolated periplasmic region of McpX ($McpX^{PR}$ and $McpX_{34-306}$) with QACs. Isothermal titration calorimetry experiments revealed tight binding to $McpX^{PR}$ with dissociation constants (K_d) in the nanomolar range for choline and glycine betaine, micromolar K_d for stachydrine and trigonelline and a K_d in the millimolar range for betonicine. Our discovery of *S. meliloti* chemotaxis to plant-derived QACs adds another role to this group of compounds, which are known to serve as nutrient sources, osmoprotectants and cell-to-cell signalling molecules. This is the first report of a chemoreceptor that mediates QACs taxis through direct binding.

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Introduction

Bacteria of the *Rhizobiaceae* family have the ability to form a species-specific mutualism with plants of the *Leguminosae* family. This intimate relationship takes place inside of the legume root, specifically in a plant organ called the nodule (Cooper, 2007; Jones *et al.*, 2007; Suzaki *et al.*, 2015). During nodule formation, the rhizobia undergo metamorphosis into bacteroids, which then fix atmospheric nitrogen into ammonium. This form of nitrogen is utilised by the plant to aid in abundant growth (Hirsch *et al.*, 2001; Jones *et al.*, 2007). Bacterial chemotaxis precedes the mutualism and enables cells to actively swim through the soil by responding to host-released compounds and accumulate in the spermosphere and rhizosphere (Ames and Bergman, 1981; Soby and Bergman, 1983; Bergman *et al.*, 1988; Caetano-Anolles *et al.*, 1988; Malek, 1989; Dharmatilake and Bauer, 1992; Uren, 2000; Scharf *et al.*, 2016). Rhizobial motility and chemotaxis have been recognized to improve symbiotic interactions of *Bradyhizobium japonicum*, *Rhizobium leguminosarum*, *Rhizobium trifolii* and *Sinorhizobium meliloti*, with soybean, pea, clover and alfalfa, respectively (Ames and Bergman, 1981; Mellor *et al.*, 1987; Caetano-Anolles *et al.*, 1988; Miller *et al.*, 2007; Althabegoiti *et al.*, 2008).

Germinating seeds exude numerous compounds into the soil creating a species-specific spermosphere (Nelson, 2004). Seed exudates include a large variety of metabolites such as amino acids, organic acids, sugars, lipids and flavonoids (Barbour *et al.*, 1991; Nelson, 2004; Webb *et al.*, 2014). Chemotaxis towards host seed exudates have been described for *B. japonicum*, *R. leguminosarum* and *S. meliloti* (Gaworzewska and Carlile, 1982; Barbour *et al.*, 1991; Webb *et al.*, 2014; Webb *et al.*, 2016). However, the complex composition of exudates hampers the identification of individual molecules shaping the chemotactic response.

Attractants for flagellar-mediated bacterial chemotaxis are generally perceived by Methyl accepting Chemotaxis Proteins (MCPs). *S. meliloti* uses eight chemoreceptors, namely seven methyl-accepting chemotaxis proteins (McpT to McpZ) and one internal chemotaxis protein

(IcpA) (Meier *et al*., 2007). Each chemoreceptor is composed of a distinctive sensing domain and a highly conserved signalling domain. Six of the MCPs are localized to the cytoplasmic membrane via two transmembrane-spanning regions, whereas McpY and IcpA lack such transmembrane domains (Meier *et al*., 2007). A variety of molecules have been recognized to serve as chemoattractants for *S. meliloti*, including amino acids, organic acids and sugars, but the cognate chemoreceptors are mostly unidentified (Meier *et al*., 2007). Through behavioural and *in vitro* binding assays, we recently discovered that *S. meliloti* McpU mediates proline chemotaxis via direct binding (Webb *et al*., 2014). McpU was also found to be an important sensor for exudates from germinating alfalfa seeds and for the amino acid portion of exudates (Webb *et al*., 2016). The periplasmic region of McpU ($McpU^{PR}$) is predicted to have a double Cache domain (dCache) (Upadhyay *et al*., 2016). Cache domains generally bind small molecules, such as amino acids (Anantharaman and Aravind, 2000; Anantharaman *et al*., 2001; Zhulin *et al*., 2003) and can bind at least two ligands, one in each Cache domain. Mutational analyses and molecular modeling showed that ligands bind to the amino proximal Cache domain of McpU (Webb *et al*., 2014). A second *S. meliloti* chemoreceptor predicted to have a dCache domain is McpX. However, cognate ligands for McpX or any of the remaining six chemoreceptors are unidentified.

One group of metabolites exuded by plant seeds and roots are quaternary ammonium compounds (QACs) (Phillips *et al*., 1995). This compound class includes betaines, which possess a positively charged ammonium cation that bears no hydrogen atom and a negatively charged carboxylate and are therefore zwitterionic, and the positively charged choline, which is a precursor of glycine betaine. Many QACs are prevalent in organisms and serve as nutrient sources, osmoprotectants and cell-to-cell signalling molecules (Chambers and Kunin, 1987; Kunin *et al*., 1992; Phillips *et al*., 1992; Lever *et al*., 1994; Phillips *et al*., 1998). Examples of betaines detected in plant tissues include betonicine (hydroxyproline betaine), glycine betaine, stachydrine (proline betaine) and trigonelline (Fig. 1). Stachydrine and trigonelline have been shown to be released from germinating alfalfa seeds in quantities of 1.1 and 2.3 nmol per seed, respectively (Phillips *et al*., 1992). Like flavonoids, stachydrine and trigonelline have been described to induce *S. meliloti nodD2* gene activity, which activates expression of genes necessary for nodulation with a legume host (Phillips *et al*., 1992). In *S. meliloti*, QACs can serve as nutrient sources as well as osmoprotectants and even aid in colonization of alfalfa seedling roots (Boivin *et al*., 1990; Boncompagni *et al*., 1999; Phillips *et al*., 1998; Gouffi *et al*., 2000; Barra *et al*., 2006). Moreover, chemoattraction of phytoplankton and marine bacterial species to glycine betaine has been reported to play a role in the marine microbial food web (Seymour *et al*., 2010). Finally, the archaeon *Halobacterium salinarum* is attracted to glycine betaine, choline and carnitine (Kokoeva *et al*., 2002). The study implicated an indirect sensing mechanism via a periplasmic binding protein forming a complex with these quaternary amines, which then binds to a transmembrane MCP (Kokoeva *et al*., 2002).

In this study, we quantified QACs (Fig. 1) exuded from germinating host and non-host seeds, analysed the chemotactic behaviour of *S. meliloti* towards individual QACs, identified the cognate chemoreceptor, McpX and its *in vitro* binding characteristics to QACs. This is the first report of a bacterial chemoreceptor that mediates chemotaxis to QACs through direct binding.

Results

Quaternary ammonium compound (QAC) quantification in seed exudates

We previously found the attractant proline and other amino acids to be exuded by host seeds and identified the chemoreceptor McpU as an amino acid sensor (Webb *et al*., 2014; Webb *et al*., 2016). In a quest to find new potential attractants, we performed a general LC–MS screen using hydrophilic interaction chromatography (HILIC) in positive ion mode, evaluating the seed exudates of *S. meliloti* host alfalfa (*Medicago sativa*) and closely related non-host, spotted medic (*Medicago*

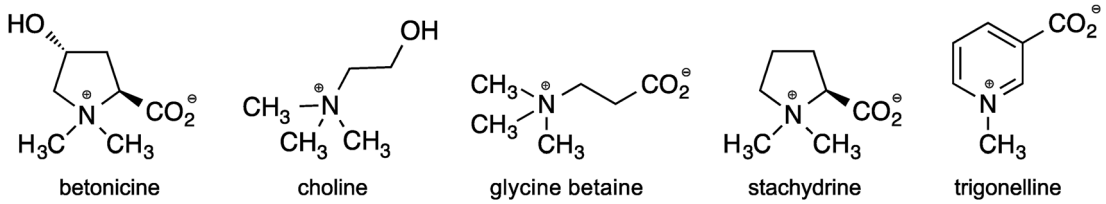

Fig. 1. Structures of quaternary ammonium compounds (QACs) analysed in this study. Betonicine and stachydrine are also known as hydroxyproline betaine and proline betaine, respectively. Of the QACs, betonicine, glycine betaine, stachydrine and trigonelline are subclassified as betaines.

arabica). This work led to the identification of several quaternary ammonium compounds (QACs) in alfalfa and spotted medic seed exudates including betonicine, choline, glycine betaine, stachydrine and trigonelline. Stachydrine and trigonelline were already known to be exuded by alfalfa seeds (Phillips *et al*., 1992; Phillips *et al*., 1995). A preliminary chemotaxis screen revealed a positive response to several QACs for *S. meliloti* wild type (data not shown), prompting the development of a method to quantify the amounts of QACs exuded by germinating seeds of alfalfa and spotted medic by LC–MS/MS (Table 1).

The QAC profiles for alfalfa and spotted medic seed exudates were different, with a total amount of 241 nmol (249 ng) and 225 nmol (221 ng) QACs, respectively. To predict the concentration of QACs at the seed surface, and therefore a value meaningful for chemotaxis experiments, exuded amounts per seed were converted using experimentally acquired seed volumes of 2.17 and 1.84 μl for alfalfa and spotted medic, respectively (Webb *et al*., 2016). Concentrations at the seed surface are predicted under the assumption that compounds will diffuse from the seed into the spermosphere, where they are detected by *S. meliloti*. Concentrations calculated to reside at the seed surface are depicted in Fig. 2 and range from approximately 4 to 400 μM, which are relevant concentrations for bacterial chemotaxis (Mesibov and Adler, 1972; Ordal and Gibson, 1977; Meier *et al*., 2007; Mello and Tu, 2007; Webb *et al*., 2014). Choline was exuded at the highest concentration and in similar amounts by both legume seeds. Stachydrine was exuded at a similar concentration by alfalfa seeds, but fourfold less by spotted medic seeds. Trigonelline was released at about half of the concentration determined for choline. Glycine betaine was exuded at 10-fold higher concentrations by spotted medic than alfalfa, but about fourfold less than choline. Finally, betonicine release was much lower than the other measured compounds for alfalfa and not detectable for spotted medic. In comparison, glycine and proline, which are structurally similar to glycine betaine and stachydrine, are exuded at approximately fivefold and 2.5-fold higher concentrations from alfalfa and spotted medic, respectively, when compared to choline (Webb *et al*., 2016). Therefore, QACs are exuded from host and non-host seeds in chemotactically relevant concentrations and with distinct profiles.

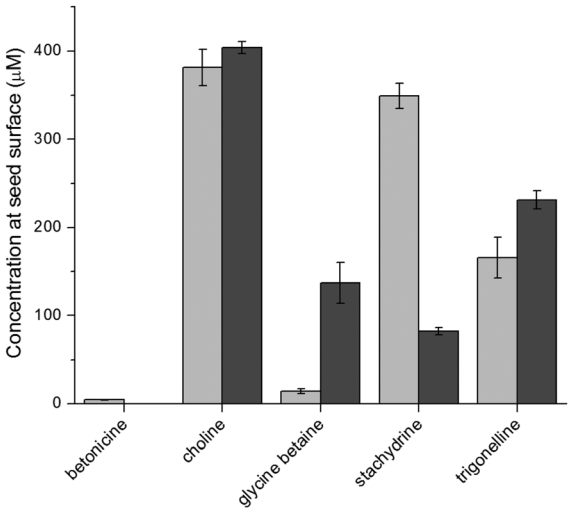

Fig. 2. QAC concentrations residing at the surface of a germinating seed. QAC quantities in seed exudates were measured with liquid chromatography–mass spectrometry. Exuded amounts and seed volumes, 2.17 μl for alfalfa and 1.84 μl for spotted medic, were used to calculate a concentration at the seed surface (Webb *et al*., 2016). Light grey bars are for an *M. sativa* seed, and dark grey bars are for an *M. arabica* seed. Values are mean and standard deviation of three biological replicates.

Table 1. Amount of QACs exuded per seed of alfalfa and spotted medic.

	ng/seed		pmol/seed	
Compound	Alfalfa	Spotted medic	Alfalfa	Spotted medic
Betonicine	1.5 ± 0.1	ND*	10 ± 0.6	ND*
Choline	86.2 ± 4.7	91.3 ± 1.5	779 ± 45	893 ± 15
Glycine betaine	3.6 ± 0.7	35.2 ± 5.9	31 ± 6	298 ± 50
Stachydrine	108.5 ± 4.4	25.6 ± 1.3	758 ± 31	179 ± 9
Trigonelline	49.2 ± 6.9	68.7 ± 3.0	827 ± 50	876 ± 22

All compounds were measured in ng ml^{-1} of exudate and converted to ng/seed or pmol/seed based on the number seeds in 0.1 g for each species. Each value is the mean of three experiments and standard deviation of the mean.
*Below the limit of detection.

Chemotaxis of S. meliloti *wild type towards QACs*

Since QACs are exuded at concentrations relevant to bacterial chemotaxis, we investigated their chemotactic potency using the Adler capillary assay. We determined concentration–response curves for the five QACs in comparison with proline. The wild-type strain RU11/001 was attracted to all QACs with a maximal response at 1 mM choline and 100 mM for the remaining four QACs (Fig. 3). Responses were highest for stachydrine, followed by glycine betaine, trigonelline and betonicine. Maximal response to choline was fourfold lower than that to stachydrine. Compared to the wild-type response to proline with a maximal response at 10 mM, stachydrine and glycine betaine elicited a stronger response, while responses to trigonelline and betonicine were comparable. No response of the chemotaxis-negative

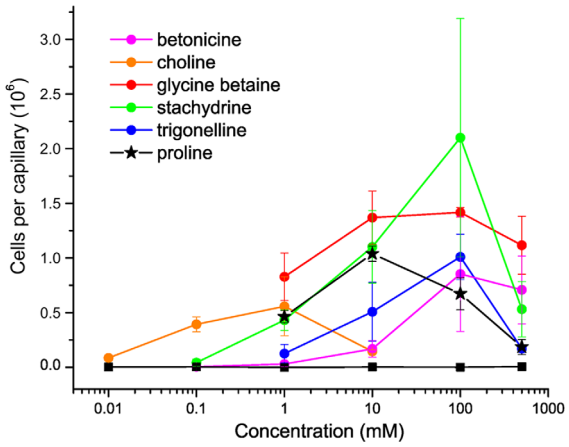

Fig. 3. Chemotaxis responses of *S. meliloti* wild type to QACs and proline in the Adler capillary assay.
Graphs with circles denote the wild-type responses to QACs; the black graph with asterisks as symbol denote the wild-type responses to proline; the black graph with squares as symbol denote the average responses of the chemotaxis null strain (*che*) to stachydrine. The standard deviation for the *che* strain is smaller than the symbols. Values are the mean and standard deviation of three biological replicates.

strain (RU13/149; *che* strain) to stachydrine was observed (Fig. 3), and the same result was obtained for betonicine (data not shown). Altogether, the QACs elicit positive chemotactic responses from *S. meliloti* wild type. Choline is a weaker attractant, but elicits a response at lower concentrations than the other QACs tested.

Identification of chemoreceptors involved in QAC sensing

To identify the chemoreceptor(s) involved in QAC sensing, we used the semi-quantitative chemotaxis drop assay to screen *S. meliloti* wild type, eight single chemoreceptor deletion mutants and a non-chemotactic *che* strain. In this assay, the accumulation of bacteria around the site of an attractant drop is observed as the formation of a denser cloud or ring, and responses were semi-quantified by comparison of pixel intensity using ImageJ. Two amino acids, glycine and proline were also included in the assay due to their structural similarities to glycine betaine and stachydrine. While the *che* strain showed no chemotactic response to any of the compounds (Table 2), the wild type displayed a strong positive response to all QACs except for betonicine, which elicited a moderate response. The majority of the strains showed a behaviour similar to wild type. Most profoundly, the Δ*mcpX* strain exhibited no response to any of the betaines and only a weak response to choline. It should be noted that the Δ*icpA* strain displayed weaker responses to all QACs and both amino acids as compared to wild type, but that responses were still more pronounced than those of the Δ*mcpX* strain. In conclusion, this screen clearly identified McpX as a chemoreceptor for QACs and indicated some involvement of IcpA. Since IcpA is assumed to be an energy sensor that senses intracellular metabolites and, therefore, is thought to mediate chemotaxis indirectly, we focused our studies on McpX.

QACs and proline interact with the periplasmic region of McpX (McpX^{PR})

Chemoattractants can interact directly with the periplasmic region of chemoreceptors or they bind to a periplasmic binding protein, which then interacts with the chemoreceptor to elicit a response (Zhang *et al.*, 1999). To test for potential interactions of the QACs with the isolated sensing domain, we overexpressed and purified the periplasmic region of McpX (McpX^{PR}, McpX$_{34\text{-}306}$) fused N-terminally with a His$_6$ tag by affinity and size exclusion chromatography. Next, we performed Differential Scanning Fluorimetry (DSF), which allows the identification of ligands that interact with and stabilize purified proteins (Niesen *et al.*, 2007;

Table 2. Chemotactic responses of *S. meliloti* strains with QACs and amino acids.

	Betonicine	Choline	Glycine betaine	Stachydrine	Trigonelline	Glycine	Proline
Wt	+	++	++	++	++	++	++
Δ*icpA*	−	+	+	+	+	+	+
Δ*mcpT*	+	+	++	++	++	++	++
Δ*mcpU*	++	−	++	++	++	−	++
Δ*mcpV*	+	++	++	++	++	++	++
Δ*mcpW*	+	+	++	+	++	+	++
Δ*mcpX*	−	+	−	−	−	++	+
Δ*mcpY*	++	++	++	++	++	++	++
Δ*mcpZ*	+	++	++	++	++	++	++
che	−	−	−	−	−	−	−

One microlitre of 100 mM attractant solution was spotted into the centre of a culture containing 0.2% hydroxypropyl methylcellulose. Chemotaxis responses were observed as accumulation of bacteria around the site of attractant drop. Images were taken after 25–30 min and change in pixel intensities was determined. − denotes no chemotactic response (changes <1), + denotes moderate chemotactic response (changes 1–4), and ++ denotes strong chemotactic response (changes >4).

Webb *et al*., 2014). The transition midpoint, T_m, during thermal unfolding of proteins shifts to higher temperatures upon binding of a low-molecular weight ligand. The T_m of McpX^{PR} was determined to be 45.0°C in the absence of a ligand and increased in the presence of all QACs (Fig. 4A), indicating that they stabilize McpX^{PR} through direct interaction. Betonicine caused the smallest shift (1°C), while the addition of choline and glycine betaine resulted in larger shifts of 10°C. Furthermore, we observed a dose response effect of McpX^{PR} stability at 10-fold lower concentrations of QACs, in particular, T_m shifts were reduced by 0.5–3.5°C (data not shown). We also screened for interactions with all proteinogenic amino acids and found that only proline caused a significant shift in T_m. Large negative shifts were observed for the acidic amino acids aspartate and glutamate. However, destabilisation is not typically observed upon ligand binding, and because the buffering capacity of the system was greater than the ligand concentration, we attribute the reduction of T_m to a likely change in net charge of the protein due to structural re-arrangements caused by binding of these amino acids. In summary, QACs and proline appear to directly interact with the sensing domain of McpX.

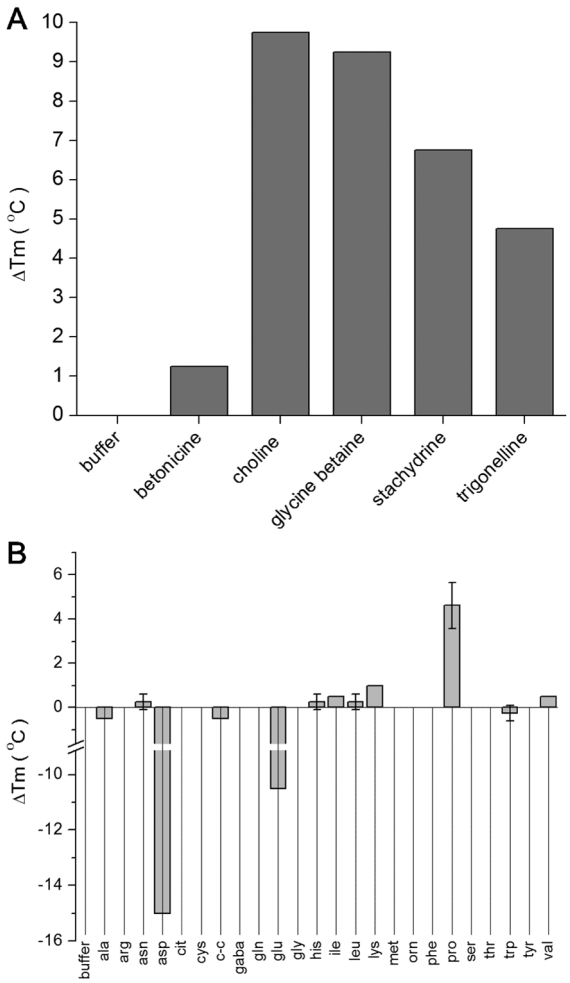

Fig. 4. T_m shifts of McpX^{PR} induced by the presence of QACs (A) and amino acids (B) measured with differential scanning fluorimetry. Protein stability was monitored as a function of fluorescence intensity and the T_m shift was recorded after subtraction of the negative control (protein alone in buffer). No standard deviation was recorded for (A), because duplicate samples displayed no variation in T_m.

Quantification of QAC chemotaxis in *S. meliloti* wild type and the *mcpX* deletion strain

The semi-quantitative drop assay and the DSF analysis identified McpX as a chemoreceptor for QACs (Table 2 and Fig. 4). To quantify the importance of McpX for QAC chemotaxis, the responses of wild type and the Δ*mcpX* strain were compared in the high-throughput quantitative hydrogel capillary assay, which monitors responses in real-time under pseudo dark field microscopy. In this assay, a hydrogel capillary containing attractant is submersed in a bacterial pond, the region around the mouth of the capillary is imaged as cells accumulate at the source of attractant, and the increase in pixel intensity caused by the accumulation of cells is quantified. Capillaries were observed over a period of 60 minutes allowing for the observation of cell accumulation due to attraction, as well as cell dissipation most likely due to depletion of the attractants from the capillary into the bacterial pond. The wild type displayed a peak in response for betonicine, choline, glycine betaine, stachydrine and trigonelline at 30, 50, 35, 20 and 19 min, respectively (Fig. 5A–E). The Δ*mcpX* strain showed no response when exposed to choline, glycine betaine, or trigonelline. However, positive responses were observed for betonicine and stachydrine with peak responses reaching approximately 60 and 40% of the wild-type peak responses, respectively (Fig. 5F). This assay confirmed McpX as a QAC receptor and revealed its indispensability for *S. meliloti* chemotaxis to choline, glycine betaine and trigonelline and its important contribution to betonicine and stachydrine chemotaxis.

QACs bind directly to McpX^{PR}

To determine binding parameters of McpX^{PR} with the QACs and proline, we performed isothermal calorimetry at 15°C using a MicroCal VP-ITC. Titrations with all compounds produced exothermic heat signals until saturation confirming direct binding (Fig. 6). Data was fit with a one binding site model for all titrations and the dissociation constants (K_d) are calculated to be 2.3 mM with betonicine, 138 nM with choline, 1.3 μM with glycine betaine, 45.2 μM with proline, 3.8 μM with stachydrine and 88.5 μM with trigonelline. Here, choline

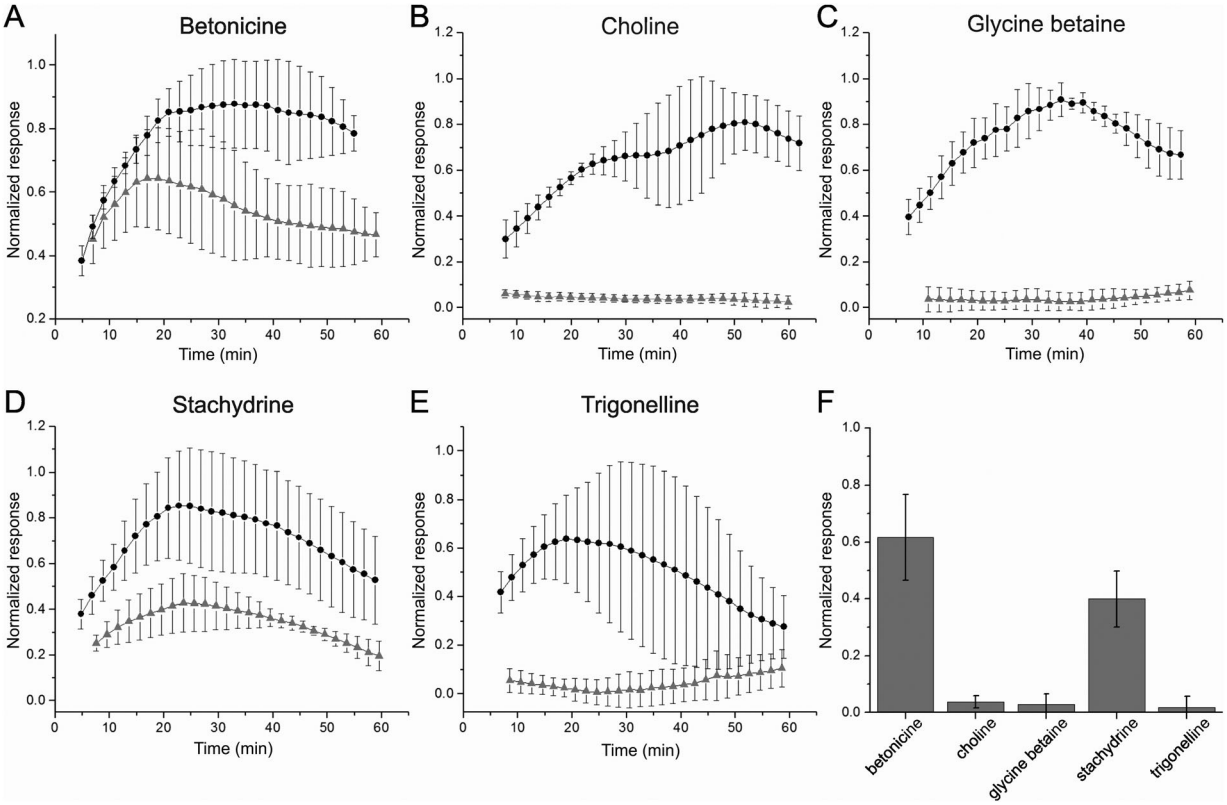

Fig. 5. Chemotaxis responses of *S. meliloti* wild type and the *mcpX* deletion strain to QACs in the hydrogel capillary assay. Images were taken under pseudo dark field, encapsulating the mouth of the capillary, and pixel intensities caused by cell accumulation were analysed. Black circles denote the wild type and gray triangles denote the *mcpX* deletion strain. Responses to (A) 50 mM betonicine; (B) 1 mM choline; (C) 1 mM glycine betaine; (D) 50 mM stachydrine; and (E) 50 mM trigonelline. (F) Responses of the *mcpX* deletion strain were normalized to the corresponding peak responses of the wild type. Briefly, the response values from five time points enveloping the peak response of the wild type were compared to the values of the same time points of the *mcpX* deletion strain. For each QAC, responses were normalized to the highest observed response of the wild type.

exhibits the strongest affinity to $McpX^{PR}$, followed by glycine betaine, stachydrine, proline, trigonelline and betonicine. The stoichiometry (ligand:protein) is approximately 1:1 for choline, proline and stachydrine, 0.6:1 for trigonelline and 2.3:1 for glycine betaine. The stoichiometry of betonicine to protein is calculated to be 0.2:1. It should be noted that the stoichiometries for trigonelline and betonicine are extrapolated from hyperbolic curves and are not as reliably accurate as the stoichiometries derived from the sigmoidal curves for the other ligands due to a lack of inflection point. Together, the ITC data confirmed results gained from the DSF experiments, proved direct binding of QACs to $McpX^{PR}$ and allowed ranking of compounds by affinity.

Discussion

Plant root exudates mediate root-rhizosphere signalling and therefore shape soil microbial communities (Badri and Vivanco, 2009). The interaction of legumes with their bacterial symbiont is initiated by the release of attractants from host-plant germinating seeds and roots, followed by directed movement of the rhizobacterium towards the plant-borne attractants. We aim to classify host plant-derived attractants and their cognate chemoreceptors in the model organism *S. meliloti* to enhance our knowledge of this important symbiotic relationship. Previously, we established that the chemoreceptor McpU mediates chemotaxis to proline and other plant-borne amino acids (Webb *et al.*, 2014; Webb *et al.*, 2016). Here, we analysed the release of quaternary ammonium compounds (QACs) by legume seeds and characterised chemotaxis of *S. meliloti* towards QACs through behavioural and *in vitro* binding assays.

Our findings demonstrate that the QACs betonicine, choline, glycine betaine, stachydrine and trigonelline are exuded by germinating seeds of alfalfa and spotted medic. We also identified homostachydrine in the seed exudates of alfalfa and spotted medic (data not shown). However, it was not quantified in this study due to the lack of a molecular standard. While stachydrine and

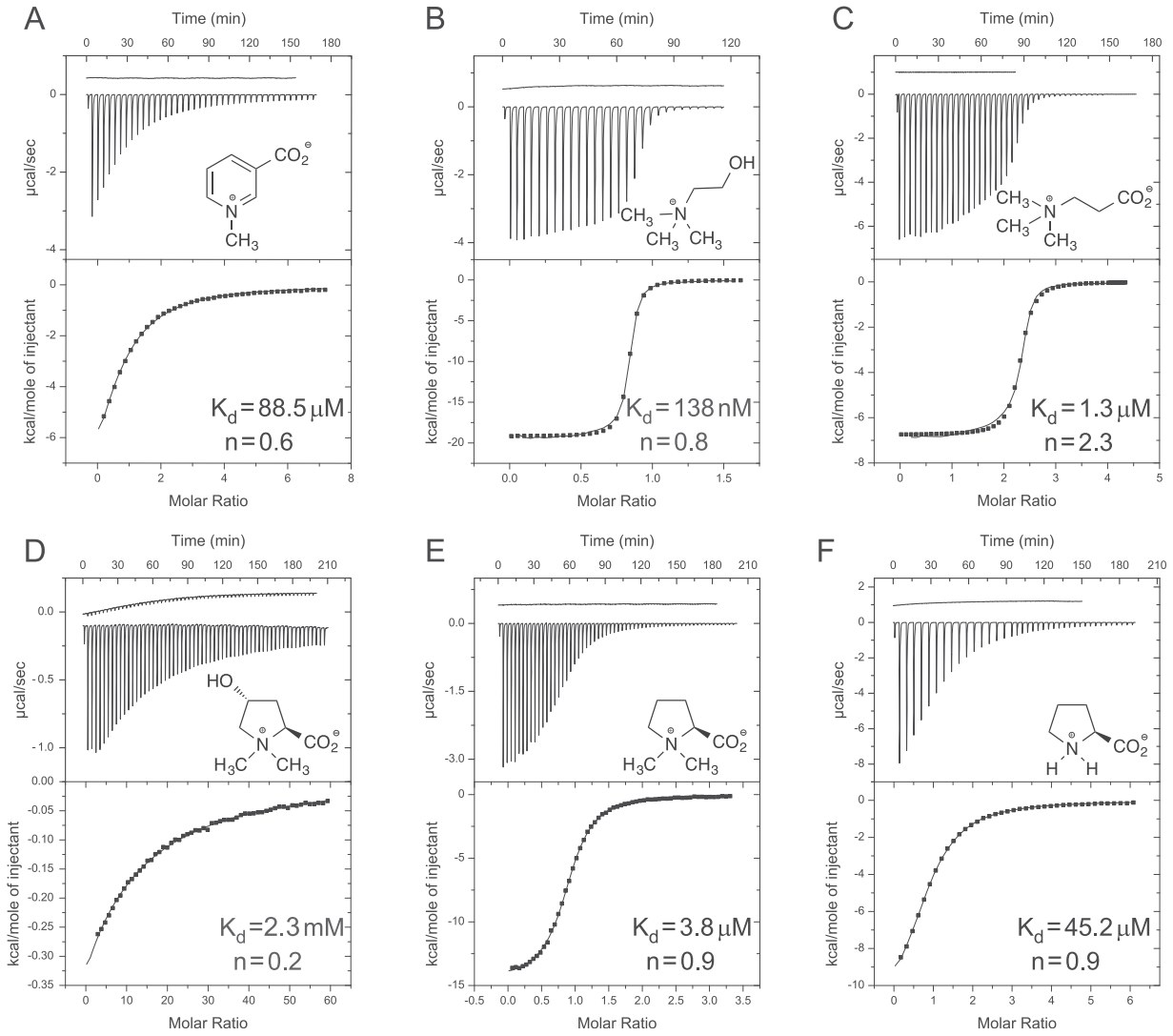

Fig. 6. Isothermal titration calorimetry of $McpX^{PR}$ with QACs and proline. Upper panels show the raw titration data, and lower panels show the normalized and dilution corrected integrated peak areas of the raw titration data. (A) A total of 75.7 µM $McpX^{PR}$ with 4.44 mM trigonelline; (B) 91.5 µM $McpX^{PR}$ with 1.5 mM choline; (C) 183 µM $McpX^{PR}$ with 5.1 mM glycine betaine; (D) 91.5 µM $McpX^{PR}$ with 20 mM betonicine; (E) 80 µM $McpX^{PR}$ with 1.5 mM stachydrine; and (F) 142 µM $McpX^{PR}$ with 6 mM proline. Data were fit with the one-set-of-sites model of the MicroCal version of Origin7 (Northampton, MA). Chemical structures represent the ligand. K_d was calculated from the reported K_a, and the *n* value is the ratio of ligand:protein.

trigonelline release from alfalfa seeds has been reported previously (Phillips *et al*., 1992; Phillips *et al*., 1995), the release of betonicine, choline and glycine betaine is a new finding. We determined that a single seed of either species exudes a total of over 2 nmol QACs, which results in concentrations of individual compounds on the seed surface of up to 400 µM. As a comparison, the structurally similar amino acids, proline and glycine, yield seed surface concentrations of 1 and 2 mM, respectively (Webb *et al*., 2016). Choline, a precursor of glycine betaine, is released in highest concentration and similar amounts from both legume seeds. The other four QACs, which are all betaines, exhibit more distinct profiles. For example, stachydrine is the betaine exuded in the highest concentration from alfalfa seeds and has been known to be the most abundant betaine in alfalfa tissues (Trinchant *et al*., 2004). It can be speculated that differences in release profiles between host and non-host seeds contribute to a specific attraction of *S. meliloti* to the alfalfa rhizosphere. The amounts of betaines in legume tissues differ between species. While alfalfa tissues have been shown to contain betonicine, homostachydrine (pipecolate betaine) and stachydrine, the closely related red clover (*Trifolium pratense*) lacks homostachydrine and stachydrine, but contains higher levels of glycine betaine and trigonelline (Wood *et al*., 1991).

As microbes adjust to the changes in osmolarity and salinity of an environment, they accumulate compatible solutes (Wood, 1999). Betaines, choline and certain amino acids such as proline are compatible solutes that protect bacterial cellular components and contribute to osmotic homeostasis and growth, thus serving as osmoprotectants (McNeil *et al*., 1999; Gouffi *et al*., 2000; Moe, 2013). Compatible solutes are utilised to various extents by different bacterial species. In *S. meliloti*, betaines are the best osmoprotectants, proline serves as an intermediate osmoprotectant, while choline is not used (Smith *et al*., 1988; Boncompagni *et al*., 1999; Alloing *et al*., 2006; Barra *et al*., 2006). Interestingly, there appears to be a positive correlation between the osmoprotectant qualities and chemoattractant strength in *S. meliloti*. In the Adler capillary assay (Fig. 3), betaines attracted a larger number of *S. meliloti* cells as compared to proline and even more so to choline, although higher concentrations of betaines were required to elicit the larger response. The dose-response curves for betonicine, stachydrine and trigonelline increased steeply to their peak at 100 mM, whereas response curves elicited by choline and glycine betaine were rather broad. This behaviour correlates with the optimal attractant concentrations used in the hydrogel assay, with the latter two QACs (choline and glycine betaine) being assayed at 1 mM, compared to 50 mM for the remaining QACs (Fig. 5). Here, we do not consider choline to be a strong attractant, because it attracts less bacteria in the capillaries than other compounds. However, *S. meliloti* is more sensitive to choline, because chemotaxis responses to choline are observed at relatively lower concentrations.

In the hydrogel capillary assay, the Δ*mcpX* strain lacked a response to choline, glycine betaine and trigonelline, but only diminished its response to betonicine and stachydrine by 40 and 60%, respectively, indicating that other receptors contribute to QAC sensing. It should be noted that during testing for optimal QAC concentrations in the hydrogel capillary assay, higher concentrations of choline and glycine betaine (up to 75 mM) did not elicit a response from the Δ*mcpX* strain (data not shown). A strong candidate for the second QAC receptor is IcpA, because the *icpA* deletion strain was the only other strain that displayed a reduced response to all QACs in the drop assay (Table 2). We are currently testing this notion by creating a Δ*icpA* Δ*mcpX* strain and assaying for QAC chemotaxis. In addition, the Δ*icpA* strain showed a reduced response to glycine and proline. The broad attractant spectrum of IcpA supports our hypothesis that IcpA serves as energy sensor, measuring the metabolic state of the cell (Meier *et al*., 2007). In fact, metabolism by *S. meliloti* has been shown for several QACs (Goldmann *et al*., 1991; Boncompagni *et al*., 1999; Burnet *et al*., 2000).

It would be interesting to investigate whether the *S. meliloti*-alfalfa symbiosis could be enhanced by QAC overproduction in the host plant and subsequent increased attraction of the symbiont to the rhizosphere of its host. Elevated glycine betaine levels have been generated in tomato plants by transformation with a bacterial *codA* gene that encodes choline oxidase to catalyze the conversion of choline to glycine betaine. This procedure yielded accumulation of glycine betaine resulting in greater tolerance to high temperatures and chilling stress during seed germination and plant growth (Park *et al*., 2004; Li *et al*., 2011). Furthermore, transgenic *Arabidopsis thaliana* expressing *codA* or two cyanobacterial glycine *N*-methyltransferases exhibited elevated levels of glycine betaine leading to improved abiotic stress tolerance (Waditee *et al*., 2005; Huang *et al*., 2008). In addition, traditional plant breeding yielded corn plants with an approximately 400-fold increase of glycine betaine in leaves (Rhodes *et al*., 1989). Much work has been done to selectively breed alfalfa for growth in high saline soils, especially to generate seeds that tolerate high salinity during germination (Bhardwaj *et al*., 2010; Anower *et al*., 2013). It would be interesting to explore whether high-salinity resistance alfalfa breeds are expressing higher QAC levels and simultaneously attracting a higher number of *S. meliloti* cells to their rhizosphere. In conclusion, enhanced production and release of compatible solutes by alfalfa can mediate high-salinity resistance to host and microsymbiont, attracts the symbiont to its host, protects it from high salt conditions and propagates symbiosis.

Results from the ITC experiments demonstrated direct binding of the periplasmic region of McpX ($MxpX^{PR}$) to all QACs and to proline. These findings are in agreement with the behavioural assays, supporting the conclusion that McpX is a QAC chemoreceptor mediating response through direct binding. The binding affinities obtained from the ITC studies correlate well with the size of the thermal shifts observed in DSF assays. In particular, choline ($K_d = 138$ nM) produced the largest shift in DSF, while addition of betonicine ($K_d = 2.3$ mM) resulted in the smallest significant shift (Fig. 5). There also appears to be a correlation between the binding strength of QACs (Figs. 4 and 6), *S. meliloti* chemotaxis response (Figs. 3 and 5), and amounts released from germinating seeds (Table 1 and Fig. 2). Choline, which yielded the highest concentrations on the seed surface, displayed the strongest binding in the ITC assay, and elicited a chemotaxis response at concentrations as low as 0.1 and 1.0 mM. In contrast, betonicine, which produced a nearly 100-fold lower concentration on the seed surface, has a 10^6fold lower affinity to $McpX^{PR}$ and elicited a maximal chemotaxis response at 100-fold higher concentration as compared to choline. Thus, it is

conceivable to speculate that McpX has evolved to display higher affinities for the more abundant QACs released by its host plant.

Titrations of $McpX^{PR}$ with choline and glycine betaine were fitted with the one-binding site model, however, these titrations yielded isotherms with two transitions. In both isotherms, the first transition is shallow and the second transition is steep. We can only speculate what causes the occurrence of two transitions. Either the ligand binds to two different sites in a cooperative manner, or $McpX^{PR}$ exists in different populations with varying affinities to the ligand. It is worth noticing that the two ligands that induce two transitions can rotate around the alpha carbon axis, whereas the other compounds tested cannot. The molecular mobility of choline and glycine betaine may influence their binding to $McpX^{PR}$.

In search for potential residues that form the ligand-binding pocket, a homology model of $McpX^{PR}$ was constructed in SWISS-MODEL using the crystal structure of the extracellular domain of $mmHK1_S$-Z3, a putative histidine kinase from *Methanosarcina mazei* (PDB ID, 3lib.1.A), as a scaffold, (data not shown). The model revealed that $McpX^{PR}$ likely has a double Cache domain (dCache) and would therefore be a member of the dCache containing family of chemoreceptors, which are known to bind small amine containing ligands (Upadhyay *et al*., 2016). However, this is the first report of a dCache containing chemoreceptor that binds quaternary amines. We are currently attempting to analyse the binding mechanism by crystallisation and X-ray diffraction data collection of $McpX^{PR}$ complexed with QAC ligands.

This study characterised the first bacterial MCP that senses QACs and determined the environmental role for *S. meliloti* chemotaxis to QACs. Overall, McpX mediates chemotaxis to QACs released into the soil during seed germination through direct ligand binding.

Experimental procedures

Bacterial strains and plasmids

Escherichia coli strains and derivatives of *S. meliloti* MV II-1 (Kamberger, 1979) and the plasmids used are listed in Table S1.

Medicago *spp*

M. sativa cultivar 'Guardsman II' (Registration number CV-203, PI 639220; Althabegoiti *et al*., 2008) and *M. arabica* (L.) Huds. (accession SA7746) seeds were used in this study.

Chemicals

Stachydrine (L-proline betaine) and betonicine (L-hydroxyproline betaine) were purchased from Extrasynthese (Toulouse, France), and choline, glycine betaine and trigonelline were from Sigma-Fluka (St. Louis, MO, USA). Amino acids were from a Fluka Analytical kit, 21 L-amino acids + glycine. Compounds were dissolved in RB and titrated to pH 7.0 with KOH for behavioural assays. For *in vitro* assays, compounds were dissolved in 100 mM NaCl, 50 mM HEPES pH 7.0, and titrated with KOH when necessary.

Media and growth conditions

E. coli strains were grown in lysogeny broth (LB) (Bertani, 1951) at 37°C. *S. meliloti* strains were grown in TYC (0.5% (wt/vol) tryptone, 0.3% (wt/vol) yeast extract, 0.087% $CaCl_2{\cdot}2H_2O$ (wt/vol) (pH 7.0) at 30°C (Platzer *et al*., 1997). Motile cells for Adler capillary assays and hydrogel capillary assays were prepared essentially as described in Webb *et al*. (2016) with minor modifications. Briefly, overnight were diluted 1:1,000 in 10 ml Rhizobial Basal minimal medium (RB) (6.1 mM K_2HPO_4, 3.9 mM KH_2PO_4, 1 mM $MgSO_4$, 1 mM $(NH_4)_2SO_4$, 0.1 mM $CaCl_2$, 0.1 mM NaCl, 0.01 mM Na_2MoO_4, 0.001 mM $FeSO_4$, 20 μg l^{-1} biotin, 1 mg l^{-1} thiamine; Götz *et al*., 1982) and layered on Bromfield agar plates (Sourjik and Schmitt, 1996). Cultures were harvested at an optical density of 600 nm (OD_{600}) of 0.16 ± 0.02. Motile cells for drop assays were prepared the same way on Bromfield-RB overlay plates, except they were grown to an OD_{600} of 0.33 ± 0.01.

Quantification of quaternary ammonium compounds (QACs) in seed exudates

Seed exudates from seeds (0.1 g) were prepared in triplicate for each *Medicago* spp. as described previously (Webb *et al*., 2016). A multiple reaction monitoring (MRM) method using authentic standards was developed for the identification and quantification of each QAC essentially as described previously (Li *et al*., 2010; Naresh Chary *et al*., 2012; Sánchez-Hernández *et al*., 2012; Servillo *et al*., 2016) using the direct infusion method on a 3200 QTrap (ABSciex, see Supporting Information for details). All samples and standards were dissolved in RB and diluted to working concentrations in 90% Buffer A (ACN:50 mM ammonium formate, pH 3.2, 9:1) and 10% Buffer B (ACN:50 mM ammonium formate, pH 3.2:water, 5:4:1), sonicated for 5 min, and centrifuged at 14,000 × *g* for 5 min. The sample queue was arranged in a random fashion with RB blank samples on each end and one in the middle. All biological samples were tested in biological triplicates and technical triplicates and standards were tested in technical triplicates. Using an Agilent 1100 series auto sampler, 5 μl of each sample was injected onto a 2.6 μm HILIC 100A HPLC column 100 × 2.1 mm (Kinetex) equipped with a KrudKatcher Ultra (Phenomenex) guard system. The column was equilibrated with 90% Buffer A and 10% Buffer B for 13 min at a flow rate of 200 μl min^{-1}, followed by sample injection and 2 min wash step. Compounds were eluted in a 9-min gradient to 100% Buffer B, followed by 2 min wash of 100% Buffer B. Ion suppression or enhancement was evident for the detection of all QACs except for

stachdyrine in alfalfa exudate. Ion suppression was accounted for by using the standard addition method (Furey *et al.*, 2013). LC–MS data were processed and analysed with Analyst (AB Sciex, Version 1.6).

Chemotaxis drop assay

Hydroxypropyl methylcellulose (HPMC) was added to a final concentration of 0.2% to each *S. meliloti* culture (in RB), and 1 ml was pipetted into a 35 mm petri plate. One microlitre of a 100 mM compound solution in RB was spotted in the centre, and plates were imaged every 2 min for 30 min in a Bio-Rad Universal Hood II with standardized camera zoom and constant exposure of 0.65 s. For each data set, images were imported into ImageJ as an image stack and analysed by setting a 25 pixel diameter circle as 'Region Of Interest (ROI)' around the centre of the plate. The *z*-axis profile of each ROI was plotted for the stack, and the average pixel intensity of the first image was subtracted from the average pixel intensity of the image with the highest intensity. The resulting change in average pixel intensity was used to determine the chemotactic response. Responses with a change in pixel intensity of less than 1 were binned into no response (−), changes of 1 to 4 were binned as moderate chemotactic response (+) and changes greater than 4 were categorized as strong chemotactic response (++). The *che* strain served as the negative control.

Traditional Adler capillary assay

Capillary assays were performed essentially as described by Adler (1973), with minor modifications (Götz and Schmitt, 1987; Meier *et al.*, 2007). Cells were harvested by centrifugation at 3,000 × *g* for 5 min at room temperature and suspended in RB to OD_{600} of 0.17. Closed U-shaped tubes (bent from 65 mm micropipettes; Drummond Scientific Co., Broomall, PA) were placed between two glass plates. For each capillary, 375 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 QAC solution. The capillaries were inserted open end first into the bacterial pond and incubated for two h at 22.5°C. Capillaries were removed, the sealed end was cut off and the complete contents were transferred into 999 μl RB using a Drummond bulb dispenser. Dilutions were plated in duplicates on TYC plates containing streptomycin. After incubation for three days at 30°C, colonies were counted. Compounds were tested in technical triplicate and the experiments were carried out in biological triplicate.

Hydrogel capillary assay

Capillaries containing a cross-linked hydrogel were prepared according to Webb *et al.* (2016). Prior to experiments, hydrogel capillaries were equilibrated for 8 h with RB with one buffer exchange after the first 4 h. For equilibration of the capillaries with QACs, capillaries were placed into 50 μl of QAC solution per capillary and incubated overnight at 4°C. Motile cells were prepared and harvested by centrifugation at 4,000 × *g* for 5 min at room temperature and suspended in RB to an OD_{600} of 0.12. Three repetitions of the hydrogel capillary assay were performed as described (Webb *et al.*, 2016). Dose responses for each QAC were performed with wild type and the Δ*mcpX* strain to determine the concentration for optimal imaging of the chemotactic responses. Requirements were an increase in pixel intensity above background, pixel intensity values below the pixel saturation value of 255, and responses that could be completely encapsulated by a drawn 'Region Of Interest (ROI)' across the observed image. The optimal concentrations were determined to be 1 mM for choline and glycine betaine and 50 mM for betonicine, stachydrine and trigonelline.

Quantification of the responses were essentially performed according to Webb et al. (2016) with minor modifications. Images from the hydrogel capillary assay were imported to MATLAB (MathWorks), and an Enhanced Correlation Coefficient (ECC) algorithm from the Image Alignment Toolbox (IAT) was utilised to perform a Euclidean transformation of images to align the capillaries in such a way that they rest precisely on top of one another when images were stacked (Evangelidis and Psarakis, 2008; Evangelidis, 2013). Rotated images were then imported to ImageJ (Rasband) as an image sequence. A rectangular region of interest (ROI) spanning 448 pixels wide and 270 pixels high was placed in front of the mouth of the capillary to encapsulate the chemotactic response and the Time Series Analyzer V3 plugin (Balaji, 2014) was utilised to attain the total intensity from this ROI (Response ROI) for each image. To account for background, an ROI with the same dimensions was placed at the top of each image, distant from the chemotactic response (Background ROI). The total intensities obtained from the background of each image were subtracted from their respective intensities of the Response ROIs. These intensity values were then normalized to the greatest total intensity value observed in the comparisons of wild type versus the Δ*mcpX* strain.

Construction of McpX^{PR} overexpressing plasmid

The *mcpX* 100–919 bps fragment was PCR amplified with Phusion DNA polymerase (NEBiolabs) using chromosomal DNA at template and cloned into Qiagen expression vector pQE30 using *Bam*HI and *Hin*dIII sites. Confirmation was obtained by pQE30 specific oligonucleotides and DNA sequencing.

Expression and purification of the periplasmic region of McpX

The recombinant ligand-binding, periplasmic region of McpX (McpX^{PR} and $McpX_{34\text{-}306}$) was overproduced from plasmid pBS455 in *E. coli* M15/pREP4, providing N-terminal His_6-tagged protein. Four litres of cell culture were grown to an OD_{600} of 0.7 at 37°C in LB containing 100 μg ml^{-1} ampicillin and 50 μg ml^{-1} kanamycin and gene

expression was induced by the addition of 0.6 mM isopropyl-β-D-thiogalactopyranoside. Cultivation was continued for 4 h at 25°C and cells were harvested and stored at −30°C. Cells were suspended in 70 ml column buffer (500 mM NaCl, 25 mM imidazole, 20 mM $NaPO_4$, pH 7.4, 2 mM tri(2-carboxyethyl)phosphine (TCEP), 1 mM phenylmethylsulfonyl fluoride [PMSF]) with 1 μg ml^{-1} of DNase and lysed by three passages through a French pressure cell at 20,000 psi (SLM Aminco, Silver Spring, MD). The soluble fraction was loaded onto three stacked 5 ml NTA columns (GE Healthcare Life Sciences) charged with Ni^{2+}. Protein was eluted from the column a linear gradient of elution buffer (500 mM NaCl, 350 mM imidazole, 20 mM $NaPO_4$, pH 7.0, 2 mM TCEP, 1 mM PMSF). Protein-containing fractions were pooled and further purified by Äktaprime™ Plus gel filtration on HiPrep 26/60 Sephacryl S-300 HR (GE Healthcare). The column was equilibrated and developed in 100 mM NaCl, 50 mM HEPES, pH 7.0 at 0.5 ml min^{-1}. Protein-containing fractions were pooled, concentrated by ultrafiltration using 10-kDa regenerated cellulose membranes in a 50 ml Amicon filter unit (Millipore, Bedford, MA) and stored at 4°C.

Thermal denaturation studies

Differential scanning fluorimetry (DSF) experiments were performed essentially as described in Webb *et al*. (2014) using a Bio-Rad CFX96 Realtime System, C1000™ Thermal Cycler in conjunction with Bio-Rad CFX Manager Software (Life Science Research 2000, Hercules, CA). Compounds were dissolved in 100 mM NaCl, 50 mM HEPES, pH 7.0 and used at final concentrations of 10 mM unless otherwise stated. $McpX^{PR}$ and SYPRO® Orange (Invitrogen, Grand Island, NY) were diluted in the same buffer to final concentrations of 10 μM protein and 0.7 × SYPRO® Orange (from 5,000 × stock). QACs were tested at 1 and 10 mM final concentrations, while amino acids were tested at a final concentration of 10 mM with the following exceptions due to solubility limitations: asn, 9.4 mM; asp, 4.8 mM; c-c, 0.115 mM; glu, 4.3 mM; phe, 6.0 mM; trp, 5.8 mM and tyr, 0.62 mM. Abbreviations are as follows, cit, citrulline; c-c, cystine; gaba, gamma aminobutyric acid; orn, ornithine. Thirty-microlitre reactions of all conditions were performed in duplicate. A temperature gradient was applied from 10 to 85°C with a 30-s equilibration at each 0.5°C step. Fluorescence was quantified using the preset FRET parameters (excitation, 490 nm; emission, 530 nm). Melting temperatures were recorded and averaged.

Isothermal titration calorimetry

$McpX^{PR}$ in 100 mM NaCl, 50 mM HEPES, pH 7.0 was used at 183 μM for testing with glycine betaine, 142 μM for testing with proline, 91.5 μM for testing with choline and trigonelline, 80 μM for testing with stachydrine, 75.7 μM for testing with betonicine, and 80 μM for the competition titration with trigonelline and glycine betaine. Ligands were dissolved in spent dialysis buffer (100 mM NaCl, 50 mM HEPES, pH 7.0). Measurements were made on a VP-ITC Microcalorimeter (MicroCal, Northampton, MA) at 15°C. $McpX^{PR}$ was placed in the sample cell and titrated with ligand. Final QAC concentrations were as follows: choline and stachydrine at 1.5 mM; betonicine at 20 mM; proline at 6 mM; glycine betaine at 5.1 mM for the direct titration and 2.22 mM for the competition titration; trigonelline at 4.4 mM for the direct titration and 446 μM for the competition titration. Baselines were produced using the compounds dissolved in dialysis buffer and were subtracted from each protein titration. For the baseline titration accompanying the competition titration, the cell was filled with trigonelline dissolved in buffer and titrated with glycine betaine. Data analysis was carried with the MicroCal version of Origin 8.1 software using the 'one binding sites' model (OriginLab, Northampton, MA).

Acknowledgements

This study was supported by National Science Foundation grant MCB-1253234. We are grateful to Jody Jervis for sample preparation, Sherry Hildreth for LC–MS analysis and Madeline Blake for help with drop assays. We are indebted to Bahareh Behkam for sharing the Zeiss Axio Observer Research microscope and the Omnicure S1000 UV light source, Florian Schubot for sharing the ABI7300 real-time PCR system and Pecetti Luciano for *M. arabica* (L.) Huds. seeds.

Author contributions

BAW, KKC and RCS conducted the experimental studies and analysed the data. BAW, WKR and RFH developed the multiple reaction monitoring (MRM) method for the proper QAC identification. TA developed the script for hydrogel capillary image analysis. BAW and BES designed the experiments, interpreted the results, and wrote the article.

References

Adler, J. (1973) A method for measuring chemotaxis and use of the method to determine optimum conditions for chemotaxis by *Escherichia coli*. *J Gen Microbiol* **74**: 77–91.

Alloing, G., Travers, I., Sagot, B., Le Rudulier, D., and Dupont, L. (2006) Proline betaine uptake in *Sinorhizobium meliloti*: characterization of Prb, an opp-like ABC transporter regulated by both proline betaine and salinity stress. *J Bacteriol* **188**: 6308–6317.

Althabegoiti, M.J., Lopez-Garcia, S.L., Piccinetti, C., Mongiardini, E.J., Perez-Gimenez, J., Quelas, J.I., Perticari, A., and Lodeiro, A.R. (2008) Strain selection for improvement of *Bradyrhizobium japonicum* competitiveness for nodulation of soybean. *FEMS Microbiol Lett* **282**: 115–123.

Ames, P., and Bergman, K. (1981) Competitive advantage provided by bacterial motility in the formation of nodules by *Rhizobium meliloti*. *J Bacteriol* **148**: 728–908.

Anantharaman, V., and Aravind, L. (2000) Cache – a signaling domain common to animal Ca(2$^+$)-channel subunits and a class of prokaryotic chemotaxis receptors. *Trends Biochem Sci* **25**: 535–537.

Anantharaman, V., Koonin, E.V., and Aravind, L. (2001) Regulatory potential, phyletic distribution and evolution of ancient, intracellular small-molecule-binding domains. *J Mol Biol* **307**: 1271–1292.

Anower, M.R., Mott, I.W., Peel, M.D., and Wu, Y. (2013) Characterization of physiological responses of two alfalfa half-sib families with improved salt tolerance. *Plant Physiol Biochem* **71**: 103–111.

Badri, D.V., and Vivanco, J.M. (2009) Regulation and function of root exudates. *Plant Cell Environ* **32**: 666–681.

Balaji, J. (2014) *Time Series Analyzer Version 3.0*. Department of Neurobiology, UCLA.

Barbour, W.M., Hattermann, D.R., and Stacey, G. (1991) Chemotaxis of *Bradyrhizobium japonicum* to soybean exudates. *Appl Environ Microbiol* **57**: 2635–2639.

Barra, L., Fontenelle, C., Ermel, G., Trautwetter, A., Walker, G.C., and Blanco, C. (2006) Interrelations between glycine betaine catabolism and methionine biosynthesis in *Sinorhizobium meliloti* strain 102F34. *J Bacteriol* **188**: 7195–7204.

Bergman, K., M., Gulash-Hoffee, R.E., Hovestadt, R.C., Larosiliere, P.G., Ronco, 2nd, L. and Su, (1988) Physiology of behavioral mutants of *Rhizobium meliloti*: evidence for a dual chemotaxis pathway. *J Bacteriol* **170**: 3249–3254.

Bertani, G. (1951) Studies on lysogenesis. I. The mode of phage liberation by lysogenic *Escherichia coli*. *J Bacteriol* **62**: 293–300.

Bhardwaj, S., Sharma, N.K., Srivastava, P.K., and Shukla, G. (2010) Salt tolerance assessment in alfalfa (*Medicago sativa* L.) ecotypes. *Bot Res J* **3**: 1–6.

Boivin, C., Camut, S., Malpica, C.A., Truchet, G., and Rosenberg, C. (1990) *Rhizobium meliloti* genes encoding catabolism of trigonelline are induced under symbiotic conditions. *Plant Cell* **2**: 1157–1170.

Boncompagni, E., Osteras, M., Poggi, M.C., and Le Rudulier, D. (1999) Occurrence of choline and glycine betaine uptake and metabolism in the family *Rhizobiaceae* and their roles in osmoprotection. *Appl Environ Microbiol* **65**: 2072–2077.

Burnet, M.W., Goldmann, A., Message, B., Drong, R., El Amrani, A., Loreau, O., Slightom, J., and Tepfer, D. (2000) The stachydrine catabolism region in *Sinorhizobium meliloti* encodes a multi-enzyme complex similar to the xenobiotic degrading systems in other bacteria. *Gene* **244**: 151–161.

Caetano-Anolles, G., Wall, L.G., De Micheli, A.T., Macchi, E.M., Bauer, W.D., and Favelukes, G. (1988) Role of motility and chemotaxis in efficiency of nodulation by *Rhizobium meliloti*. *Plant Physiol* **86**: 1228–1235.

Chambers, S.T., and Kunin, C.M. (1987) Isolation of glycine betaine and proline betaine from human urine. Assessment of their role as osmoprotective agents for bacteria and the kidney. *J Clin Invest* **79**: 731–737.

Cooper, J.E. (2007) Early interactions between legumes and rhizobia: disclosing complexity in a molecular dialogue. *J Appl Microbiol* **103**: 1355–1365.

Dharmatilake, A.J., and Bauer, W.D. (1992) Chemotaxis of *Rhizobium meliloti* towards nodulation gene-inducing compounds from alfalfa roots. *Appl Environ Microbiol* **58**: 1153–1158.

Evangelidis, G.D. (2013) *IAT: A Matlab Toolbox for Image Alignment*. http://www.iatool.net.

Evangelidis, G.D., and Psarakis, E.Z. (2008) Parametric image alignment using enhanced correlation coefficient maximization. *IEEE Trans Pattern Anal Mach Intell* **30**: 1858–1865.

Furey, A., Moriarty, M., Bane, V., Kinsella, B., and Lehane, M. (2013) Ion suppression; a critical review on causes, evaluation, prevention and applications. *Talanta* **115**: 104–122.

Gaworzewska, E.T., and Carlile, M.J. (1982) Positive chemotaxis of *Rhizobium leguminosarum* and other bacteria towards root exudates from legumes and other plants. *J Gen Microbiol* **128**: 1179–1188.

Goldmann, A., Boivin, C., Fleury, V., Message, B., Lecoeur, L., Maille, M., and Tepfer, D. (1991) Betaine use by rhizosphere bacteria: genes essential for trigonelline, stachydrine, and carnitine catabolism in *Rhizobium meliloti* are located on pSym in the symbiotic region. *Mol Plant Microbe Interact* **4**: 571–578.

Götz, R., Limmer, N., Ober, K., and Schmitt, R. (1982) Motility and chemotaxis in two strains of *Rhizobium* with complex flagella. *J Gen Microbiol* **128**: 789–798.

Götz, R., and Schmitt, R. (1987) *Rhizobium meliloti* swims by unidirectional, intermittent rotation of right-handed flagellar helices. *J Bacteriol* **169**: 3146–3150.

Gouffi, K., Bernard, T., and Blanco, C. (2000) Osmoprotection by pipecolic acid in *Sinorhizobium meliloti*: specific effects of D and L isomers. *Appl Environ Microbiol* **66**: 2358–2364.

Hirsch, A.M., Lum, M.R., and Downie, J.A. (2001) What makes the rhizobia-legume symbiosis so special? *Plant Physiol* **127**: 1484–1492.

Huang, J., Rozwadowski, K., Bhinu, V.S., Schafer, U., and Hannoufa, A. (2008) Manipulation of sinapine, choline and betaine accumulation in *Arabidopsis* seed: towards improving the nutritional value of the meal and enhancing the seedling performance under environmental stresses in oilseed crops. *Plant Physiol Biochem* **46**: 647–654.

Jones, K.M., Kobayashi, H., Davies, B.W., Taga, M.E., and Walker, G.C. (2007) How rhizobial symbionts invade plants: the *Sinorhizobium-Medicago* model. *Nat Rev Microbiol* **5**: 619–633.

Kamberger, W. (1979) An Ouchterlony double diffusion study on the interaction between legume lectins and rhizobial cell surface antigens. *Arch Microbiol* **121**: 83–90.

Kokoeva, M.V., Storch, K.F., Klein, C., and Oesterhelt, D. (2002) A novel mode of sensory transduction in archaea: binding protein- mediated chemotaxis towards osmoprotectants and amino acids. *EMBO J* **21**: 2312–2322.

Kunin, C.M., Hua, T.H., Van Arsdale White, L., and Villarejo, M. (1992) Growth of *Escherichia coli* in human urine: role of salt tolerance and accumulation of glycine betaine. *J Infect Dis* **166**: 1311–1315.

Lever, M., Sizeland, P.C., Bason, L.M., Hayman, C.M., and Chambers, S.T. (1994) Glycine betaine and proline betaine in human blood and urine. *Biochim Biophys Acta* **1200**: 259–264.

Li, C., Hill, R.W., and Jones, A.D. (2010) Determination of betaine metabolites and dimethylsulfoniopropionate in coral tissues using liquid chromatography-time-of-flight mass spectrometry and stable isotope-labeled internal standards. *J Chromatogr B: Analyt Technol Biomed Life Sci* **878**: 1809–1816.

Li, S., Li, F., Wang, J., Zhang, W., Meng, Q., Chen, T.H., Murata, N., and Yang, X. (2011) Glycinebetaine enhances the tolerance of tomato plants to high temperature during germination of seeds and growth of seedlings. *Plant Cell Environ* **34**: 1931–1943.

Malek, W. (1989) Chemotaxis in *Rhizobium meliloti* strain L5.30. *Microbiology* **152**: 611–612.

McNeil, S.D., Nuccio, M.L., and Hanson, A.D. (1999) Betaines and related osmoprotectants. Targets for metabolic engineering of stress resistance. *Plant Physiol* **120**: 945–950.

Meier, V.M., Muschler, P., and Scharf, B.E. (2007) Functional analysis of nine putative chemoreceptor proteins in *Sinorhizobium meliloti*. *J Bacteriol* **189**: 1816–1826.

Mello, B.A., and Tu, Y. (2007) Effects of adaptation in maintaining high sensitivity over a wide range of backgrounds for *Escherichia coli* chemotaxis. *Biophys J* **92**: 2329–2337.

Mellor, H.Y., Glenn, A.R., Arwas, R., and Dilworth, M.J. (1987) Symbiotic and competitive properties of motility mutants of *Rhizobium trifolii* Ta1. *Arch Microbiol* **148**: 34–39.

Mesibov, R., and Adler, J. (1972) Chemotaxis toward amino acids in *Escherichia coli*. *J Bacteriol* **112**: 315–326.

Miller, L.D., Yost, C.K., Hynes, M.F., and Alexandre, G. (2007) The major chemotaxis gene cluster of *Rhizobium leguminosarum* bv. *viciae* is essential for competitive nodulation. *Mol Microbiol* **63**: 348–362.

Moe, L.A. (2013) Amino acids in the rhizosphere: from plants to microbes. *Am J Bot* **100**: 1692–1705.

Naresh Chary, V., Dinesh Kumar, C., Vairamani, M., and Prabhakar, S. (2012) Characterization of amino acid-derived betaines by electrospray ionization tandem mass spectrometry. *J Mass Spectrom* **47**: 79–88.

Nelson, E.B. (2004) Microbial dynamics and interactions in the spermosphere. *Annu Rev Phytopathol* **42**: 271–309.

Niesen, F.H., Berglund, H., and Vedadi, M. (2007) The use of differential scanning fluorimetry to detect ligand interactions that promote protein stability. *Nat Protoc* **2**: 2212–2221.

Ordal, G.W., and Gibson, K.J. (1977) Chemotaxis toward amino acids by *Bacillus subtilis*. *J Bacteriol* **129**: 151–155.

Park, E.J., Jeknic, Z., Sakamoto, A., DeNoma, J., Yuwansiri, R., Murata, N., and Chen, T.H. (2004) Genetic engineering of glycinebetaine synthesis in tomato protects seeds, plants, and flowers from chilling damage. *Plant J Cell Mol Biol* **40**: 474–487.

Phillips, D.A., Joseph, C.M., and Maxwell, C.A. (1992) Trigonelline and stachydrine released from alfalfa seeds activate NodD2 protein in *Rhizobium meliloti*. *Plant Physiol* **99**: 1526–1531.

Phillips, D.A., Sande, E.S., Vriezen, J.A.C., de Bruijn, F.J., Rudulier, D.L., and Joseph, C.M. (1998) A new genetic locus in *Sinorhizobium meliloti* is involved in stachydrine utilization. *Appl Environ Microbiol* **64**: 3954–3960.

Phillips, D.A., Wery, J., Joseph, C.M., Jones, A.D., and Teuber, L.R. (1995) Release of flavonoids and betaines from seeds of seven *Medicago* species. *Crop Sci* **35**: 805–808.

Platzer, J., Sterr, W., Hausmann, M., and Schmitt, R. (1997) Three genes of a motility operon and their role in flagellar rotary speed variation in *Rhizobium meliloti*. *J Bacteriol* **179**: 6391–6399.

Rhodes, D., Rich, P.J., Brunk, D.G., Ju, G.C., Rhodes, J.C., Pauly, M.H., and Hansen, L.A. (1989) Development of two isogenic sweet corn hybrids differing for glycinebetaine content. *Plant Physiol* **91**: 1112–1121.

Sánchez-Hernández, L., Nozal, L., Marina, M.L., and Crego, A.L. (2012) Determination of nonprotein amino acids and betaines in vegetable oils by flow injection triple-quadrupole tandem mass spectrometry: a screening method for the detection of adulterations of olive oils. *J Agric Food Chem* **60**: 896–903.

Scharf, B.E., Hynes, M.F., and Alexandre, G.M. (2016) Chemotaxis signaling systems in model beneficial plant-bacteria associations. *Plant Mol Biol* **90**: 549–559.

Servillo, L., Giovane, A., Casale, R., Balestrieri, M.L., Cautela, D., Paolucci, M., Siano, F., Volpe, M.G., and Castaldo, D. (2016) Betaines and related ammonium compounds in chestnut (*Castanea sativa* Mill.). *Food Chem* **196**: 1301–1309.

Seymour, J.R., Simo, R., Ahmed, T., and Stocker, R. (2010) Chemoattraction to dimethylsulfoniopropionate throughout the marine microbial food web. *Science* **329**: 342–345.

Smith, L.T., Pocard, J.A., Bernard, T., and Le Rudulier, D. (1988) Osmotic control of glycine betaine biosynthesis and degradation in *Rhizobium meliloti*. *J Bacteriol* **170**: 3142–3149.

Soby, S., and Bergman, K. (1983) Motility and chemotaxis of *Rhizobium meliloti* in soil. *Appl Environ Microbiol* **46**: 995–998.

Sourjik, V., and Schmitt, R. (1996) Different roles of CheY1 and CheY2 in the chemotaxis of *Rhizobium meliloti*. *Mol Microbiol* **22**: 427–436.

Suzaki, T., Yoro, E., and Kawaguchi, M. (2015) Leguminous plants: inventors of root nodules to accommodate symbiotic bacteria. *Int Rev Cell Mol Biol* **316**: 111–158.

Trinchant, J.C., Boscari, A., Spennato, G., Van de Sype, G., and Le Rudulier, D. (2004) Proline betaine accumulation and metabolism in alfalfa plants under sodium chloride stress. Exploring its compartmentalization in nodules. *Plant Physiol* **135**: 1583–1594.

Upadhyay, A.A., Fleetwood, A.D., Adebali, O., Finn, R.D., and Zhulin, I.B. (2016) Cache domains that are homologous to, but different from PAS domains comprise the largest superfamily of extracellular sensors in prokaryotes. *PLoS Comput Biol* **12**: e1004862.

Uren, N.C. (2000) Types, amounts and possible functions of compounds released into the rhizosphere by soil-grown plants. In: *The Rhizosphere: Biochemistry and Organic Substances at the Soil-Plant Interface*. R. Pinton, Z. Varanini, and P. Nannipiero (eds). New York: CRC Press, pp. 19–40.

Waditee, R., Bhuiyan, M.N., Rai, V., Aoki, K., Tanaka, Y., Hibino, T., *et al.* (2005) Genes for direct methylation of glycine provide high levels of glycinebetaine and abiotic-stress tolerance in *Synechococcus* and *Arabidopsis*. *Proc Natl Acad Sci USA* **102**: 1318–1323.

Webb, B.A., Helm, R.F., Scharf, B.E. (2016) Contribution of individual chemoreceptors to *Sinorhizobium meliloti* chemotaxist towards amino acids of host and nonhost seed exudates. *Mol Plant Microbe Interact* **29**: 231–239.

Webb, B.A., Hildreth, S., Helm, R.F., Scharf, B.E. (2014) *Sinorhizobium meliloti* chemoreceptor McpU mediates chemotaxis toward host plant exudates through direct proline sensing. *Appl Environ Microbiol* **80**: 3404–3415.

Wood, J.M. (1999) Osmosensing by bacteria: signals and membrane-based sensors. *Microbiol Mol Biol Rev* **63**: 230–262.

Wood, K.V., Stringham, K.J., Smith, D.L., Volenec, J.J., Hendershot, K.L., Jackson, K.A., *et al*. (1991) Betaines of alfalfa: characterization by fast atom bombardment and desorption chemical ionization mass spectrometry. *Plant Physiol* **96**: 892–897.

Zhang, Y., Gardina, P.J., Kuebler, A.S., Kang, H.S., Christopher, J.A., Manson, M.D. (1999) Model of maltose-binding protein/chemoreceptor complex supports intrasubunit signaling mechanism. *Proc Natl Acad Sci USA* **96**: 939–944.

Zhulin, I.B., Nikolskaya, A.N., Galperin, M.Y. (2003) Common extracellular sensory domains in transmembrane receptors for diverse signal transduction pathways in bacteria and archaea. *J Bacteriol* **185**: 285–294.

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