Contribution of Individual Chemoreceptors to *Sinorhizobium meliloti* Chemotaxis Towards Amino Acids of Host and Nonhost Seed Exudates

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Plant seeds and roots exude a spectrum of molecules into the soil that attract bacteria to the spermosphere and rhizosphere, respectively. The alfalfa symbiont *Sinorhizobium meliloti* utilizes eight chemoreceptors (McpT to McpZ and IcpA) to mediate chemotaxis. Using a modified hydrogel capillary chemotaxis assay that allows data quantification and larger throughput screening, we defined the role of *S. meliloti* chemoreceptors in sensing its host, *Medicago sativa*, and a closely related nonhost, *Medicago arabica*. *S. meliloti* wild type and most single-deletion strains displayed comparable chemotaxis responses to host or nonhost seed exudate. However, while the *mcpZ* mutant responded like wild type to *M. sativa* exudate, its reaction to *M. arabica* exudate was reduced by 80%. Even though the amino acid (AA) amounts released by both plant species were similar, synthetic AA mixtures that matched exudate profiles contributed differentially to the *S. meliloti* wild-type response to *M. sativa* (23%) and *M. arabica* (37%) exudates, with McpU identified as the most important chemoreceptor for AA. Our results show that *S. meliloti* is equally attracted to host and nonhost legumes; however, AA play a greater role in attraction to *M. arabica* than to *M. sativa*, with McpZ being specifically important in sensing *M. arabica*.

The legume-rhizobia symbiosis is characterized by the infection of a legume root by rhizobia resulting in the formation of an organ called the nodule. Inside the nodule, rhizobia transform into bacteroids, which fix atmospheric nitrogen into a utilizable nitrogen source, resulting in greater legume biomass. In return, the legume provides nutrients to the rhizobia. A key step in the initiation of nodulation is the rhizobial recognition of compatible host legume-secreted flavonoids, followed by the legume recognition of compatible microsymbiont-borne Nod factors (Cooper 2007; Hirsch et al. 2001; Jones et al. 2007; van Rhijn and Vanderleyden 1995). The symbiotic partners have developed very specific means of molecular dialogue, resulting in interactions that can be highly exclusive (Garau et al. 2005; Sprent 2007). For example, the soil-dwelling bacterium *Sinorhizobium meliloti* is hosted by the agriculturally important legume *Medicago sativa* (alfalfa) (Jones et al. 2007) as well as *Melilotus alba* (white sweet clover) (Yan et al. 2000) and *Medicago truncatula* (barrel medic) (Terpolilli et al. 2008). However,

the closely related *Medicago arabica* (spotted medic) cannot serve as a host for *S. meliloti* (Garau et al. 2005).

There are several steps prior to nodulation that contribute to propagation of the symbiosis, including rhizobial chemotaxis, attachment mediated by type IVb pili, exopolysaccharide synthesis and biofilm formation, and flavonoid-Nod factor recognition (Downie 2010; Fujishige et al. 2006; González et al. 1996; Gulash et al. 1984; Kamberger 1979; Rinaudi and González 2009; Sorroche et al. 2010; Wang et al. 2012; Zatakia et al. 2014). Chemotaxis is one of the earliest steps in symbiosis, which enables rhizobia to respond to compounds released by host seed and roots and actively swim toward and accumulate in the spermosphere and rhizosphere (Ames and Bergman 1981; Bergman et al. 1988; Caetano-Anollés et al. 1988b; Dharmatilake and Bauer 1992; Malek 1989; Soby and Bergman 1983; Uren 2000). This initial step has proven to be important for successful symbiotic interactions in several rhizobia-legume partnerships including *S. meliloti*–alfalfa, *Rhizobium trifolii*–clover, and *Rhizobium leguminosarum*–pea (Ames and Bergman 1981; Caetano-Anollés et al. 1988b; Mellor et al. 1987; Miller et al. 2007). Even more, a strain of *Bradyrhizobium japonicum* with enhanced chemotaxis had a greater nodulation efficiency than the wild type (Althabegoiti et al. 2008).

A germinating seed exudes a multitude of compounds into the soil creating a spermosphere that is unique to that species and even to that seed (Miller and Oldroyd 2012; Nelson 2004). The molecular composition of seed exudate includes amino acids (AA), sugars, lipids, phenolics, proteins, and other metabolites (Barbour et al. 1991; Nelson 2004; Webb et al. 2014). Chemotaxis to their host seed exudates has been shown for *S. meliloti*, *B. japonicum*, and *R. leguminosarum* (Barbour et al. 1991; Gaworzewska and Carlile 1982; Webb et al. 2014), but knowledge of the compounds responsible is mostly lacking. A more comprehensive study of the attractants exuded by alfalfa seeds and the corresponding *S. meliloti* chemoreceptors is required for the development of strains with enhanced chemotaxis toward the host.

Previous work has characterized particular aspects of the *S. meliloti* chemotaxis system regarding the number and domain topology of chemoreceptors (Meier et al. 2007), chemoreceptor-ligand specificity (Meier et al. 2007; Webb et al. 2014), the two-component regulatory components (Amin et al. 2014; Dogra et al. 2012; Riepl et al. 2004; Sourjik and Schmitt 1998), and the unidirectional, speed-variable flagellar motor (Attmannspacher et al. 2005; Eggenhofer et al. 2004; Platzer et al. 1997; Sourjik and Schmitt 1996). *S. meliloti* has eight genes coding for putative chemoreceptors called methyl-accepting chemotaxis proteins (McpS to McpZ) and one internal chemotaxis protein (IcpA)

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lacking typical methyl-accepting sites (Galibert et al. 2001; Meier et al. 2007). McpS, encoded from a second chemotaxis operon on the pSymA plasmid, is not expressed when cells are motile and a contribution to chemotaxis can be excluded (Meier and Scharf 2009; Meier et al. 2007). The eight chemoreceptors contributing to chemotaxis share a highly conserved, C-terminal signaling domain, which forms a ternary complex with two cytoplasmic chemotaxis proteins, CheA, an autophosphorylating 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 CheY2, which interacts with the cytoplasmic face of the flagellar motor and controls the swimming paths of bacteria (Scharf 2002; Sourjik and Schmitt 1996). Seven of the *S. meliloti* MCP proteins possess two transmembrane regions and a periplasmic region harboring the ligand-binding domain located between the transmembrane helices. In contrast, McpY and IcpA lack such hydrophobic regions and, thus, exhibit a cytosolic localization (Meier et al. 2007). All the chemoreceptors vary in their ligand-binding domains; however, McpU, McpV, and McpX share specialized domains such as Cache_1 and Cache_2 signaling domains (Meier et al. 2007), which are known to bind small molecules such as AA (Anantharaman and Aravind 2000; Ulrich and Zhulin 2005). McpY possesses a PAS domain, which typically senses redox potential, oxygen, or light (Taylor and Zhulin 1999). Deletion of individual receptor genes causes differential impairments in the chemotactic response toward a variety of small biomolecules tested, including various sugars, five AA, and four organic acids (Meier et al. 2007), but a more complete study is needed to establish their significance in host sensing.

While *S. meliloti* chemotaxis mutants are less competitive in inducing nodule formation with alfalfa (Ames and Bergman 1981; Caetano-Anollés et al. 1988b), little is known about the specific role of individual chemoreceptors in chemotactic host recognition. To date, only McpU has been characterized in playing a significant role in host plant recognition by mediating chemotaxis to host-derived proline (Webb et al. 2014). To identify *S. meliloti* chemoreceptors important for host sensing, quantitative chemotaxis assays of wild type and single-receptor deletion strains were performed with *M. sativa* seed exudate. In comparison, the seed exudate of the closely related nonhost *M. arabica* was tested to identify differential responses of mutant strains and, concomitantly, differences in exudate attractant composition. Since AA are relatively strong attractants for *S. meliloti* (Götz et al. 1982; Meier et al. 2007), we also analyzed the AA profiles of host and nonhost exudates, their contribution to chemotaxis, and the involvement of individual chemoreceptors in AA sensing.

RESULTS

A modified agarose capillary assay with automated data acquisition and quantification.

The classic Adler capillary assay allows for the direct quantitative measurement of the response of a population of cells to an attractant or repellent gradient (Adler 1973). However, when several variables are to be tested, such as multiple strains or attractants, or both, this method can become tedious and time consuming. In addition, reproducible removal of the capillary contents can be difficult (Sampedro et al. 2015). The modified agarose or hydrogel capillary assays ease the assessment of chemotaxis, but the results are purely qualitative (Parales and Harwood 2002; Webb et al. 2014). To avoid disadvantages of the classic capillary assay but still generate quantitative data, we combined the simplicity of the modified hydrogel capillary assay with a quantifiable measure of the chemotactic response at the mouths of the capillaries.

The setup of the chemotaxis chambers on a microscope slide is illustrated in Figure 1A. The dimensions of the setup allow for testing of up to ten different variables, such as strains and attractants or repetitions. Once the slide is set on the microscope stage, it is important to utilize a motorized focusing drive with software that allows for memorization of the *x*, *y*, and *z* coordinates for each capillary. Figure 1B illustrates the pipeline for image acquisition and analysis for one chemotaxis chamber (details discussed below). The combination of the hydrogel capillary assay, motorized focusing drive, and image analysis software enabled quantitative time-course assessments of the chemotactic response, providing detailed comparative information on multiple experimental variables.

Chemotaxis of *S. meliloti* wild type toward seed exudate of *M. sativa* and *M. arabica*.

To assess the potencies of germinating seed exudates from *M. sativa* (host) and *M. arabica* (nonhost), the *S. meliloti* wild-type strain RU11/001 was tested with the hydrogel capillary assay for its ability to sense either exudate. In this assay, capillaries contained a hydrogel that was equilibrated with seed exudate. This format allowed efflux of liquid material but prevented cells from swimming into the capillary; thus, cells that were attracted to the exudate accumulated at the mouth of the capillary. A control experiment was performed to rule out a chemotactic response of *S. meliloti* to rhizobial basal medium (RB), used to dissolve the exudate. Comparatively, wild type and the *che* strain, which lacks all chemoreceptor genes, exhibited no response to RB, whereas wild type displayed a strong response to *M. sativa* exudate, which is abolished for the *che* strain (Fig. 2A). For quantitative analyses, photographs were taken at the mouth of the capillary every 4 min under a pseudo dark field, the pixel intensity in front of the capillaries was quantified using the time series analyzer V3 plug-in of the ImageJ software (Balaji 2014), and the intensities were normalized to the greatest intensity observed from the wild type. In this assay, pixel intensity positively correlates with the amount of cells present and, thus, with strength of the chemotactic response. To determine the optimal exudate concentration for optical observation and pixel intensity quantification, a dose-response assay was performed (data not shown). During the first 12 min, the wild type appeared to show a slightly greater response to the *M. arabica* exudate. However, after 12 min, the response to either exudate did not differ from each other (Fig. 2B). The responses plateaued between 20 and 34 min before declining. It should be noted that the plateau in the response is purely physiological, because pixel intensities did not reach a level of saturation in the images. Upon microscopic examination, it was observed that the declination in response after 34 min was due to the dissemination of cells away from the capillary. This is most likely due to depletion of the attractants from the capillary into the bacterial pond. Taken together, these results support the claim that the chemotactic potencies of *M. arabica* and *M. sativa* seed exudate are relatively equal.

Chemotaxis of single *S. meliloti* chemoreceptor deletion strains to *M. sativa* and *M. arabica* seed exudates.

To elucidate the importance of each chemoreceptor in *M. sativa* and *M. arabica* exudate sensing, eight single-deletion strains were assessed for their maximum chemotactic response, using the hydrogel capillary assay. The wild type exhibited a maximum response to exudate of *M. sativa* and *M. arabica* between 20 and 34 min (Fig. 2B); therefore, the pixel intensities of the mutant images pertaining to this time period were averaged and normalized to the greatest average intensity observed for the wild type for the same time period (Fig. 3). McpT, McpU, McpV,

McpY, and IcpA appeared to contribute similarly to the chemotaxis response toward either legume exudate. A strain lacking *mcpU* showed the most diminished response toward *M. sativa* and *M. arabica* legume exudates, with reductions to 35 and 20% of the wild-type response, respectively. Strains lacking *mcpW* and *mcpX* displayed significant reductions in response to the exudate of *M. sativa* and *M. arabica*, respectively. Interestingly, the strain lacking *mcpZ* displayed a pronounced differential response toward the two legume exudates. While this strain responded like wild type to the *M. sativa* exudate, its reaction to the *M. arabica* exudate was reduced by 80%. The results indicate that the attractant compositions of the two legume exudates are different and that *S. meliloti* senses an altered but, presumably, overlapping attractant spectrum in *M. sativa* and *M. arabica* exudates.

Chemotaxis of *S. meliloti* toward *M. sativa* and *M. arabica* seed exudates and synthetic AA mixtures.

S. meliloti is able to utilize each proteinogenic AA for growth and displays positive chemotaxis of varied strengths to all of them (Burg et al. 1982; Götz et al. 1982; Jordan 1952; Malek 1989). Therefore, we first analyzed the AA composition of *M. sativa* and *M. arabica* seed exudates to investigate their contribution to host sensing. Exudates from surface-sterilized and germinated seeds of *M. sativa* and *M. arabica* were quantified with liquid chromatography mass spectrometry (LC-MS) in conjunction with authentic AA standards. The amounts of each AA exuded by one seed was determined and the concentration of these AA at the seed surface was calculated, using the average *M. sativa* and *M. arabica* seed volumes of 2.17 and 1.84 μl , respectively. Figure 4 gives a comparison of the concentration of each AA on the seed surface of *M. sativa* or *M. arabica*. Since both legume species are closely related, there appears to be no striking differences between the amounts of each AA exuded. It should be noted that the concentration of each AA is within range of eliciting a chemotactic response, thus making the individual amounts exuded relevant for attraction of *S. meliloti* to the spermosphere and, therefore, for symbiosis with *M. sativa*. Since chemotaxis has been observed for the non-proteinogenic AA, ornithine, citrulline, and cystine (data not shown), their analysis was included in this study.

To assess the chemotactic contribution of the AA fraction in the exudate (proteinogenic AA plus cystine, ornithine, and citrulline), synthetic AA mixtures were created mimicking the exact quantities detected in the seed exudates of *M. sativa* and *M. arabica*, and the wild-type responses to the exudates were compared with the responses to their respective synthetic AA mixture. Here, responses to *M. sativa* exudate and the *M. sativa* synthetic AA mixture were normalized to the highest response observed to the exudate. The same normalization procedure was done for the *M. arabica* exudate and synthetic AA mixture. In both cases, the wild-type response toward the exudate is greater than it is toward the respective synthetic AA mixture (Fig. 5A and B). By adding up the normalized response values

for each data set, we determined that the wild-type response to the *M. sativa* and *M. arabica* synthetic AA mixtures is 23 and 37% of its response to the corresponding exudates, respectively. In conclusion, AA have a greater contribution to nonhost than to host exudate chemotaxis, indicating that host sensing is mediated by additional attractants other than the measured AA.

Fig. 1. Hydrogel capillary assay set up and flow chart of image acquisition and analysis. **A**, Ten chemotaxis chambers are constructed on a microscope slide (100 × 50 × 1 mm). Each chamber consists of an O-ring 8.5 mm in diameter obtained from the cap of a Sarstedt 1.5-ml screw-cap tube that is sealed to the slide with an application of machine grease. A bacterial suspension (81 μl) is placed within the O-ring and a 0.5- μl hydrogel capillary is slid into the chamber via a precut section of the O-ring. A glass coverslip is placed on top of the O-ring and is sealed by an application of machine grease. The chemotaxis response is considered to start when the coverslip is set in place. **B**, Upon microscopic observation of a chemotaxis chamber, the guidelines in the flow chart are followed to acquire images of the chemotaxis response and to quantify the response in each image.

Chemotaxis of single *S. meliloti* chemoreceptor deletion strains to synthetic AA mixture mimicking *M. sativa* exudate composition.

To determine the chemotactic contribution of individual chemoreceptors toward the synthetic AA mixture of the *S. meliloti* host *M. sativa*, the single-receptor deletion strains were assessed for their maximum chemotactic response, using the hydrogel capillary assay, and responses were quantified (Fig. 6). All deletion strains but $\Delta mcpY$ exhibited a decreased

response, although this reduction was not statistically significant for $\Delta mcpT$, $\Delta mcpV$, $\Delta mcpW$, and $\Delta mcpZ$. The single deletion strains $\Delta mcpX$ and $\Delta icpA$ displayed significantly reduced responses of approximately 35 and 40%, respectively, whereas a strain lacking *mcpY* exhibited a significantly increased response. Most interestingly, a strain lacking *mcpU* showed the largest reduction compared with wild type, i.e., a 90% decrease. This result may indicate that McpU is not only a receptor for proline, as previously shown (Webb et al. 2014), but proposes a function as a general AA sensor. The intermediate response levels of strains lacking *mcpT*, *mcpV*, *mcpW*, *mcpX*, and *icpA* suggests that these chemoreceptors also play a role in AA sensing.

Fig. 2. *Sinorhizobium meliloti* wild-type response to *Medicago sativa* and *M. arabica* exudates. **A**, Responses of *S. meliloti* wild type and the *che* strain to *M. sativa* seed exudate (exudate) and rhizobial basal medium (RB) were observed at 25 $\times$ magnification under pseudo dark field and images were taken at 20 min. Exudate was diluted with RB and was tested at 5 $\times$ concentration of the original exudate sample. To allow for ease of visualization, the image brightness, contrast, and intensity were adjusted equally for each image. **B**, Responses to *M. sativa* ($\circ$) and *M. arabica* ($\blacksquare$) exudates were observed at 25 $\times$ magnification under pseudo dark field, and images were taken with a 1.3-millisecond exposure every 4 min. Normalized response values are the mean and standard deviation of the pixel intensities from three independent experiments. Each response value for a strain is plotted against an average time point. Exudates were tested at 5 $\times$ concentration of the original exudate samples.

Chemotaxis to synthetic AA mixtures with and without proline.

To determine if McpU is important for chemotaxis to AA other than proline, the chemotactic responses of the wild type and the $\Delta mcpU$ strain to the synthetic AA mixture were compared with their responses to a synthetic AA mixture lacking proline. When exposed to the synthetic mixture with proline, as expected, the $\Delta mcpU$ strain exhibited a reduced response compared with the wild type (Fig. 7A and C); however, there appeared to be no difference in the responses to the synthetic mixtures without proline (Fig. 7B and D). This result implies that McpU senses AA other than proline and is likely a general AA sensor.

DISCUSSION

S. meliloti uses chemotaxis to direct its movements toward host seed and root-exuded attractants (Dharmatilake and Bauer 1992; Malek 1989). This early host-microbe interaction is important for increasing nodulation efficiency, because it allows the bacterial cells to effectively locate infection sites and compete for

Fig. 3. Responses of *Sinorhizobium meliloti* wild type and single *mcp* deletion strains to exudates of *Medicago sativa* and *M. arabica*. Responses to *M. sativa* (dark gray bars) and *M. arabica* (light gray bars) exudates were observed at 25 $\times$ magnification under pseudo dark field. The wild type exhibited a maximum response to exudates of *M. sativa* and *M. arabica* between 20 and 34 min. Therefore, images taken during this time period were averaged. Responses were normalized to the wild-type response (horizontal line at 1.0) and are the mean and standard deviation from three independent experiments. Exudates were tested at 5 $\times$ concentration of the original exudate samples. Data were analyzed for statistical significance, using the one or two sample *t* test, $P \leq 0.05$. Responses marked with an asterisk (*) are significantly smaller than the wild type, and the responses of $\Delta mcpZ$, marked with **, are significantly different toward both exudates from each other ($P \leq 0.01$).

nodulation (Ames and Bergman 1981; Gulash et al. 1984). Germinating alfalfa seeds release compounds that attract *S. meliloti*, which can be viewed as an early recruitment phase of the microsymbiont to the spermosphere. The initial recruitment and establishment of a positional advantage on the roots may be attained by attractants with a longer diffusion range, such as hydrophilic AA, organic acids, sugars, and sugar alcohols (Ma et al. 2005), whereas hydrophobic substances such as phenolic compounds might only have short-range effects. In fact, organic metabolites have been reported to be effective chemoattractants for *S. meliloti* (Burg et al. 1982; Götz et al. 1982; Meier et al. 2007; Robinson and Bauer 1993), while flavonoids, although proposed as host-specific attractants, elicit a rather small chemotactic response (Caetano-Anollés et al. 1988a; Dharmatilake and Bauer 1992). Since chemical compounds are commonly exuded by many plant species during germination, comparing the attractant profile of *S. meliloti* host and nonhost legumes can lead to a better understanding of the initial stages of symbiont recruitment.

We observed no obvious difference in the strength or time course of attraction of *S. meliloti* wild type to seed exudate of the host *M. sativa* and the closely related nonhost *M. arabica*, in the hydrogel capillary assay (Fig. 2B). This may be simply due to the high phylogenetic proximity of the two medics; however, the specific molecular composition of host and nonhost exudates is unknown. Therefore, we cannot conclude that i) the attractant spectrum is the same for both exudates or ii) *S. meliloti* will accumulate equally well in host and nonhost spermosphere and rhizosphere. To address the first point, a detailed analysis of the secretome and attractome of host versus nonhost exudate is required. The second idea can be challenged by analyzing preferential accumulation of *S. meliloti* in a microfluidic device with competing host and nonhost exudates (Sahari et al. 2014).

When we analyzed the chemotaxis response of single-receptor deletion strains to both exudates, certain parallels and differences emerged. McpU and IcpA appeared to be most important for general exudate sensing, followed by McpV, McpW, McpX, and McpY (Fig. 3). While the role of McpU in proline (Webb et al. 2014) and general AA sensing (Fig. 6) has been uncovered, little is

known about the ligand spectrum of the remaining receptors (Meier et al. 2007). In particular, a function of the cytosolic IcpA in sensing the metabolic state of the cell has been discussed (Meier et al. 2007). Most interestingly, McpZ plays no role in host sensing but is equally as important as McpU for nonhost sensing (Fig. 3). Thus, the attractants sensed by McpZ are absent in the host exudate, which is a clear indication that the attractant profiles of both exudates differ. In conclusion, *S. meliloti* senses a divergent but most likely overlapping attractant spectrum in *M. sativa* and *M. arabica* exudates.

AA are documented as potent attractants (Burg et al. 1982; Götz et al. 1982), and our previous work revealed McpU as proline sensor (Webb et al. 2014). The present study identified McpU as the most important receptor for host sensing (Fig. 3). Therefore, we quantified each proteinogenic AA, citrulline, cystine, and ornithine in the host and nonhost seed exudates, determining their millimolar concentrations per seed to classify their abundances in the spermosphere. Götz et al. (1982) specified the chemotactic thresholds for each proteinogenic AA ranging from 10^{-6} M

Fig. 4. Amino acids exuded by germinating seeds. Bars indicate the concentrations of amino acids at the surface of a germinating seed as determined by liquid chromatography-mass spectrometry for *Medicago sativa* (dark gray bar) and *M. arabica* (light gray bar). The seed volumes are 2.17 and 1.84 μ l for *M. sativa* and *M. arabica*, respectively. Data are organized from highest to lowest concentrations and are the mean and standard deviation from three biological replicates and two to four technical replicates.

Fig. 5. Wild-type responses to exudates and synthetic amino acid (AA) mixtures. **A.** *Medicago sativa* exudate (■) and synthetic *M. sativa* AA mixture (○). **B.** *M. arabica* exudate (■) and *M. arabica* synthetic AA mixture (○). To observe responses that are visibly bright enough for quantification, seed exudate and AA mixtures were tested at a 7.6 $\times$ concentration of the original exudate samples. Normalized response values are the mean and standard deviation from three independent experiments. Each response value for a strain is plotted against an average time point.

(proline) to 10^{-4} M (aspartate). Thus, single seeds from both medics exude chemotactically relevant amounts of most proteinogenic AA. The AA profiles appear very similar between the two medics, although with slightly higher amounts exuded by *M. arabica* (Fig. 4). The only significant difference between the two profiles is the concentration of alanine, which is approximately 40% higher in *M. arabica* exudate. While this difference is small, it could still be influential on the scale of chemotactic strength.

Using the hydrogel capillary assay, we observed the wild-type response to the *M. sativa* and *M. arabica* synthetic AA mixtures to be 23 and 37% of the respective exudates (Fig. 5), suggesting that non-AA attractants contribute significantly more to the chemotactic response than AA. Potential attractants based upon the metabolomes of *Medicago* seeds include nonreducing oligosaccharides, phenolics as well as betaines (Horbowicz et al. 1995; Phillips et al. 1995). Due to the presence of these compounds in the exudate, it is conceivable that they modulate chemotaxis to AA. Thus, reported contributions of the AA based upon synthetic mixtures may not directly correlate to AA-based chemotaxis in exudate samples. Studies are currently under way to identify the remaining attractants and characterize differences in the attractant profile between host and nonhost secretomes. The majority of the single deletion strains exhibited a reduced response to the synthetic *M. sativa* AA mixture, inferring that most receptors, directly or indirectly, play a role in AA sensing. Clearly, this study revealed that the proline receptor McpU also senses other AA (Figs. 6 and 7). McpU binds proline in its amino-proximal cache domain (Webb et al. 2014), and it remains to be shown whether the same holds true for other AA.

Identifying environmentally relevant chemotaxis signals in soil bacteria, in particular plant symbionts, and matching them to respective chemoreceptors is a difficult task, due to the complex composition of the rhizosphere. Additionally, bacterial chemoreceptors are known to sense chemically diverse ligands through two different mechanisms, i.e., via direct binding to the periplasmic region or indirectly involving specialized

periplasmic binding proteins. As an example, the *Escherichia coli* chemoreceptor Tar mediates positive chemotaxis to aspartate through direct binding and senses maltose through binding of maltose-bound maltose-binding protein (Kossmann et al. 1988). McpU appears to be the most important chemoreceptor for host sensing, and it remains to be seen whether it is mediating chemotaxis to attractants other than AA. Furthermore, the attractant profiles of the remaining seven *S. meliloti* chemoreceptors need to be identified to understand their role in host sensing. With this knowledge, a promising avenue for enhancement of *S. meliloti* chemotaxis toward its host would be opened. This goal could be attained by i) increased expression of receptors dedicated to host sensing, ii) genetic tuning of receptors to induce a greater positive chemotactic response toward host-specific exuded attractants, and iii) increased exudation of host-borne attractants. Fine-tuning the chemotaxis process could assist in microsymbiont positioning early during rhizosphere formation, resulting in a greater recruitment of *S. meliloti* to the plant host and, thus, greater crop yields.

MATERIALS AND METHODS

Bacterial strains and plasmids.

Derivatives of *S. meliloti* MV II-1 (Kamberger 1979) used are listed in Table 1.

Media and growth conditions.

S. meliloti strains were grown in TYC (0.5% [wt/vol] tryptone, 0.3% [wt/vol] yeast extract, 0.13% $\text{CaCl}_2 \times 6 \text{H}_2\text{O}$

Fig. 6. Responses of single *mcp* deletion strains to *Medicago sativa* synthetic amino acid mixture. Responses were observed at 25× magnification under pseudo dark field. Images taken between 20 and 34 min were averaged and responses are normalized to the wild-type response (horizontal line at 1.0). Data are the mean and standard deviation from three independent experiments. Each response value for a strain is plotted against an average time point. Exudates were tested at 7.6× concentration of the original exudate samples. Data were analyzed for statistical significance using the one sample *t* test, $P \leq 0.05$. Responses marked with an asterisk (*) are significantly smaller ($\Delta mcpU$, $\Delta mcpX$, $\Delta icpA$) or larger ($\Delta mcpY$) than the wild type.

Fig. 7. Responses of wild type and the $\Delta mcpU$ strain to *Medicago sativa* synthetic amino acid (AA) mixtures with and without proline. **A**, Wild-type response to AA mixture with proline. **B**, Wild-type response to AA mixture without proline. **C**, $\Delta mcpU$ response to AA mixture with proline. **D**, $\Delta mcpU$ response to AA mixture without proline. AA mixtures were tested at a 7.6× concentration of the original exudate samples. Responses were observed at 25× magnification under pseudo dark field and images were taken at 10 min. To allow for ease of visualization, the image brightness, contrast, and intensity were adjusted equally for each image.

[wt/vol] [pH 7.0], streptomycin [600 µg/ml]) at 30°C (Bertani 1951; Platzer et al. 1997). Motile cells for hydrogel capillary assays were grown for 2 days in TYC, were diluted 1:1,000 in 3 ml of TYC and were grown overnight. Cultures were then diluted 1:100 in 10 ml of minimal RB (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 per liter, 100 µg of thiamine per liter (Götz et al. 1982), layered on Bromfield agar plates with no antibiotics (Sourjik and Schmitt 1996), and were incubated at 30°C for approximately 15 h to an optical density at 600 nm (OD₆₀₀) of 0.17 ± 0.02.

Preparation of seed exudates.

The *Medicago sativa* cultivar Guardsman II (registration number CV-203, PI 639220) used in this 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, U.S.A. Seeds were harvested in 2012 and were stored at 4°C prior to experiments. *Medicago arabica* (L.) Huds. (accession SA7746) from a wild population in Siena, Italy in 2008, was cultivated ex situ in Lodi, Italy in 2010, and seeds were harvested and stored at 4°C prior to experiments. Seeds (0.1 g, approximately 47 ± 1 *M. sativa* seeds and 49 ± 1 *M. arabica* seeds) were placed in a 50-ml conical tube and were washed four times with 35 ml of autoclaved, sterile-filtered water. Next, seeds were washed once with 8 ml of commercial hydrogen peroxide (3% in water) for 12 min and, then, four times with 35 ml of autoclaved, sterile filtered water under slow agitation. Seeds were then transferred to a 125-ml Erlenmeyer flask containing 20 ml of autoclaved, sterile-filtered water. Seeds were jostled to distribute them evenly at the bottom of the flask and were germinated without shaking at 30°C for 24 h. To harvest the exudate, the contents were mixed by swirling, a 19-ml aliquot of seed supernatant was removed from the flask, was placed into a 50-ml conical tube, and was flash frozen in liquid nitrogen. The frozen sample was freeze-dried for approximately 48 h and stored at -20°C. Prior to flash freezing, seed exudates were tested for bacterial contamination by microscopic examination and by plating on TYC plates and observation of colony forming units after incubation at 30°C for 48 h. Contaminated samples were not included in this study. Seed exudate powders were resuspended to a volume less than the original volume in the flask, thus concentrating the contents to 50x. Aliquots were stored at -30°C and, prior to a chemotaxis assay, they were thawed and then diluted in RB to working concentrations. Postincubation, the germination efficiency was approximately 90 to 95% for both medics, as determined by radicle emergence. Seed exudate powders were resuspended to a 50x smaller volume than the original volume in the flask, thus concentrating the contents. Aliquots were stored at -30°C, were thawed prior to a chemotaxis assay, and then, were diluted in RB to working concentrations.

Quantification of AA in *M. arabica* and *M. sativa* seed exudates by LC-MS.

For each medic, seed exudate residue from six biological replicates was thawed to room temperature, was suspended in 250 µl of RB, and was sonicated for 10 min. The samples were centrifuged at 4,000 × g for 10 min to pellet insoluble material. The six biological replicates were combined in pairs, yielding three biological replicates consisting of 500 µl per combined sample. The samples were briefly vortexed and were split into 120-µl aliquots to be used for either AA quantification or chemotaxis experiments. Aliquots were stored at -20°C prior to use. Aliquots were thawed on ice, were briefly vortexed, were sonicated for 5 min, were centrifuged at 14,000 × g for 5 min to pellet any remaining insoluble material, and were transferred to

microfuge tubes that had been previously rinsed with 100% ethanol. Exudate samples were diluted 1:1, 1:3, 1:5, and 1:7 in RB, titrated to pH 3.5 with HCl, and 100 µl of material per sample was processed using the EZ:faas for free physiological amino acid analysis by LC-MS, as previously described (Webb et al. 2014). AA standard solutions, ranging from 55 to 220 nmoles per milliliter made from the Phenomenex kit (Sigma-Aldrich, St. Louis), were derivatized alongside the exudate samples to establish a calibration curve for each AA. Using Analyst software (AB Sciex, Concord, Canada) for data analysis, the peak area of the internal standard homoarginine was used to normalize the concentration of the AA Ala, Arg, Asn, Asp, Cit, Gln, Glu, Gly, His, Lys, Met, Orn, Pro, Ser, Thr, and Trp, and the peak area of the internal standard homophenylalanine was used to normalize Ile, Leu, Phe, cystine (C-C), and Tyr. The Phenomenex kit does not allow for quantification of cysteine. Results are given as the average concentration derived from three biological replicates, with two to four technical replicates, and duplicate injections per technical replicate.

Creation of synthetic AA mixtures.

Synthetic mixtures were made with AA from the Fluka Analytical kit 21 L-Amino acids + glycine (lot number 09416), which were dissolved in RB to 50x the concentrations observed in seed exudates, were aliquoted, and were stored at -30°C until ready for experiments.

Preparation of hydrogel capillaries.

Capillaries containing a cross-linked hydrogel were prepared essentially according to Webb et al. (2014) with minor modifications. Briefly, capillaries (0.5 µl of Drummond microcaps) were sealed at one end in a Bunsen burner flame and the inner glass surface was hydroxylated by placing the mouth of the capillaries into a 1% (vol/vol) solution of 3-(trichlorosilyl) propyl methacrylate (TPM) (Sigma-Aldrich) diluted in paraffin oil at room temperature for 10 min. After excess TPM was removed via desiccation, the capillaries were submerged in 100% ethanol and were put under desiccation for approximately 30 s. Ethanol was removed via desiccation and capillaries were wiped with a microfiber cloth to remove any residual material. Capillaries were then quickly passed once through a Bunsen burner flame. A 10% (wt/vol) solution of poly(ethylene glycol) diacrylate with an average M_n number average molecular weight 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 precursor solution, which was subsequently pulled into the capillaries by placing them under

Table 1. *Sinorhizobium meliloti* strains and plasmids

Strain/plasmid	Relevant characteristics ^a	Source or reference
RU11/001	Spontaneous Sm ^r wild-type strain	Pleier and Schmitt 1991
RU11/803	Sm ^r ; $\Delta mcpW$	Meier et al. 2007
RU11/804	Sm ^r ; $\Delta mcpY$	Meier et al. 2007
RU11/805	Sm ^r ; $\Delta mcpX$	Meier et al. 2007
RU11/815	Sm ^r ; $\Delta icpA$	Meier et al. 2007
RU11/818	Sm ^r ; $\Delta mcpZ$	Meier et al. 2007
RU11/828	Sm ^r ; $\Delta mcpU$	Meier et al. 2007
RU11/830	Sm ^r ; $\Delta mcpV$	Meier et al. 2007
RU11/838	Sm ^r ; $\Delta mcpT$	Meier et al. 2007
RU13/149	Sm ^r ; $\Delta mcpS$, $\Delta mcpT$, $\Delta mcpU$, $\Delta mcpV$, $\Delta mcpW$, $\Delta mcpX$, $\Delta mcpY$, $\Delta mcpZ$, $\Delta icpA$ (<i>che</i>)	Meier et al. 2007

^a Nomenclature according to Bachmann (1990). Sm^r = streptomycin-resistant.

vacuum for approximately 30 s. The mouths of the capillaries were submersed in the precursor solution, and photopolymerization was performed for 20 s, using a 365 nm, 18 W cm⁻² UV light source (Omnicure S1000; Vanier, Quebec, Canada). Hydrogel capillaries were observed under 100× magnification to check for hydrogel integrity. If any hydrogel extended from the mouth of the capillary, the mouth was gently scraped with a wet Kimwipe and was rechecked for hydrogel integrity. Only capillaries with a flush surface at the mouth were used for experiments. Hydrogel capillaries were washed with distilled water and then soaked in distilled water with 1 µg of ampicillin per milliliter, 1 µg of kanamycin per milliliter, 1 µg of neomycin per milliliter, and 1 µg of gentamicin per milliliter for at least 4 h at room temperature, followed by three subsequent washes with the same solution. Hydrogel capillaries could be stored in distilled water with antibiotics at 4°C for at least two months.

Hydrogel capillary assay.

Prior to experiments, hydrogel capillaries were washed and equilibrated twice for 4 h with RB. AA mixtures and seed exudates suspended in RB were thawed to room temperature, were briefly vortexed, were centrifuged at 13 × g for 3 min, and were diluted to working concentrations in RB. For experiments including only seed exudates, the solutions were tested at 5× concentration. For experiments including seed exudates and AA mixtures, the solutions were tested at 7.6× concentration. For experiments with only the AA mixtures, the solutions were tested at 7.6× concentration. For equilibration of the capillaries with attractant, capillaries were placed into 50 µl of attractant solution per capillary and were left overnight at 4°C. Cells were harvested by centrifugation at 4,000 × g for 5 min at room temperature and were suspended with RB to an OD₆₀₀ of 0.12, and 81 µl of each strain suspension was added to a circular chemotaxis chamber formed by a microscope slide (100 × 50 mm), a cut rubber O-ring with an inner diameter of 8.5 mm and a thickness of 1 mm (Sarstedt), and a coverslip. The open end of a hydrogel capillary was inserted into the chemotaxis chamber before the coverslip was placed on top. After the assembly of ten such chambers (Fig. 1A), the microscope slide was placed on the computer-assisted stage of a Zeiss Axio observer research microscope. By programming the Zen software module, the X, Y, and Z positions of all ten chambers were observed at 25× magnification under pseudo dark field and images were taken with a 1.3-millisecond exposure every 2 min.

Quantification of chemotaxis responses.

Images from the hydrogel capillary assay were imported to the GIMP 2.0 Image Manipulation Program (the GNU Image Manipulation Program Team) and were adjusted by rotation to align the capillaries in such a way that the capillaries rest precisely on top of one another when images were stacked. Rotated images were then imported to ImageJ as an image sequence and the time series analyzer V3 plugin (Balaji 2014) was utilized to obtain the total intensity from a rectangular region of interest (ROI) spanning 551 pixels wide and 294 pixels high. The total intensity of an ROI is reported as 'area,' but will be referred to as 'intensity' in this study. The placement of the ROI was centered in front of the mouth of the capillary to encapsulate the chemotactic response in the images. Images were maintained as .tiff files with no compression. For a single repetition, the mean intensity obtained for the chemotaxis-negative strain (*che*) at each time point was subtracted from the mean intensities obtained for all other strains. These intensity values were then normalized to the greatest average intensity value observed from the wild-type strain. In all experiments, intensity values were normalized to the highest wild-type response observed. Due to small variations in experimental start time, each area value for a strain is plotted against the average time point. An average time point was derived

from the three closest time points for an intensity value from three independent experiments.

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AUTHOR-RECOMMENDED INTERNET RESOURCE

U. S. National Institutes of Health ImageJ software:
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