Specific root exudates compounds sensed by dedicated chemoreceptors shape *Azospirillum brasilense* chemotaxis in the rhizosphere

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Abstract

Plant roots shape the rhizosphere community by secreting compounds that recruit diverse bacteria. Colonization of various plant roots by the motile alphaproteobacterium *Azospirillum brasilense* causes increased plant growth, root volume, and crop yield. Bacterial chemotaxis in this and other motile soil bacteria is critical for competitive colonization of the root surfaces. The role of chemotaxis in root surface colonization has previously been established by end-point analyses of bacterial colonization levels detected a few hours to days after inoculation. More recently, microfluidic devices have been used to study plant-microbe interactions, but these devices are size-limited. Here, we use a novel slide-in chamber that allows real-time monitoring of plant-microbe interactions using agriculturally-relevant seedlings to characterize how bacterial chemotaxis mediates plant root surface colonization during the association of *A. brasilense* with *Triticum aestivum* (wheat) and *Medicago sativa* (alfalfa) seedlings. We track *A. brasilense* accumulation in the rhizosphere and on the root surfaces of wheat and alfalfa. *A. brasilense* motile cells display distinct chemotaxis behaviors in different regions of the roots, including attractant and repellent responses that ultimately drive surface colonization patterns. We also combine these observations with real-time analyses of behaviors of wild type and mutant strains to link chemotaxis responses to distinct chemicals identified in root exudates, to specific chemoreceptors that together explain the chemotactic response of motile cells in different regions of the roots. Further, the bacterial second messenger, c-di-GMP, modulates these chemotaxis responses. Together, the findings illustrate dynamic bacterial chemotaxis responses to rhizosphere gradients that guide root surface colonization.

Importance

Plant root exudates play critical roles in shaping rhizosphere microbial communities and the ability of motile bacteria to respond to these gradients mediate competitive colonization of root surfaces. Root exudates are complex chemical mixtures that are spatially and temporally dynamic. Identifying the exact chemical(s) that mediate recruitment of soil bacteria to specific regions of the roots is thus challenging. Here, we connect patterns of bacterial chemotaxis responses and sensing by chemoreceptors to chemicals found in root exudates gradients and identify key chemical signals that shape root surface colonization in different plants and regions of the roots.

Introduction

Crop production is dependent upon plant-microbe interactions in the rhizosphere. Beneficial rhizosphere microbes may increase plant yield by producing phytohormones and fixing atmospheric nitrogen (1, 2). The rhizosphere is a highly dynamic and complex system composed of microenvironments affected by plant and microbial activities. Rhizosphere conditions determine plant health, plant productivity, and affect the overall carbon and nitrogen soil cycles (3, 4). Characterizing rhizosphere conditions remains challenging due to the high spatial and temporal dynamics and the paucity of methods to track such conditions at relevant scales, although several recent advances have been made (e. g. (5, 6)).

Motility and chemotaxis, the directed swimming in chemical gradients, guide beneficial bacteria to desirable niches, especially in the soil where bacterial chemotaxis function is enriched compared to marine and sediment environments (7, 8). Chemotaxis is initiated when chemicals in the environment bind to membrane-anchored chemotaxis receptors. A repellant-bound chemotaxis receptor causes phosphorylation of the associated cytoplasmic histidine kinase,

CheA from ATP. CheA-P then interacts with and phosphorylates CheY. CheY-P diffuses through the cytoplasm, interacts with the flagellar motor, and causes a change in the direction of rotation of the flagellar motor, which reorients the cell in a new swimming direction through a tumble or a reversal (9, 10). Chemotaxis to root exudates and to individual compounds found in these root exudates has been demonstrated in many bacterial species (8, 11-16). While insightful, many of these studies rely on single time point analyses to elucidate the role of chemotaxis proteins and chemoreceptors in sensing exudates or specific compounds within these exudates and plant colonization (11, 13, 15, 17). These methods give a rather static view of chemotaxis in complex gradients of the rhizosphere which is in contrast to the dynamic and short-lived (within seconds) nature of this bacterial behavior. The exception to this are recent studies which used a microfluidic device to monitor *Bacillus subtilis* behavior and colonization of *Arabidopsis thaliana* roots over time (5, 18). While this device allows plant-bacteria interactions to be studied in real-time, it is limited to small seedlings, which excludes many agriculturally important plants.

Previous work has shown that several plant growth promoting bacteria species (8, 11, 19-22) must colonize their plant hosts to exhibit plant growth promoting effects, but how this colonization is established is unknown (22). *Azospirillum brasilense* is one of these soil-dwelling and plant-growth promoting bacterium that can colonize a variety of plants and these bacteria are sold as commercial inoculants worldwide (15). The genome of *A. brasilense* encodes 4 chemotaxis signal transduction systems, with two of these modulating flagellar motility and controlling chemotaxis and, 51 chemotaxis receptors (23, 24). The roles of motility and chemotaxis as well as other cellular functions in root surface colonization by *A. brasilense* and related species have been previously studied using mutant strains, end-point assays and

microscopy observations (15, 25, 26). With respect to motility, these experiments established a role for the polar flagellum and flagellar motility (27, 28), some chemotaxis receptors (29-31), and chemotaxis signaling (23) in wheat (*Triticum aestivum*) root surface colonization. Similar to what is known in other motile plant-associated bacteria, the emerging picture of root surface colonization events by *A. brasilense* is relatively static because the establishment and progression of plant roots-microbe association are typically derived from discrete, fixed timepoints, often obtained from distinct samples. To address this shortcoming, we developed a slide-in chamber that allows both whole plant roots and bacteria to be monitored in real time for up to 1 week, without disrupting the interaction. This chamber allows monitoring of free-swimming bacterial behavior, colonization along the roots, and temporal changes in plant-microbe associations. Using this tool, we track *A. brasilense* accumulation in the rhizosphere and on the root surfaces of wheat and alfalfa seedlings. We identify chemotaxis attractant and repellent responses in discrete regions of roots that drive surface colonization patterns. We also combine these observations with real time behaviors and include mutant strains to link chemotaxis responses to distinct chemicals, that we identify in root exudates, to specific chemoreceptors that together explain the responses of motile cells in different regions of the roots.

Results

Slide-in chamber allows real time monitoring of plant-microbe interactions

To observe real-time plant-microbe interactions, we developed a novel slide-in chamber which allows for plants and bacteria to be monitored spatially and temporally, undisturbed, for up to 1 week using confocal microscopy (Fig S1) (32). The chamber consists of a 85 mm x 65

mm x 8 mm rectangle with a 62 mm x 20 mm rectangular opening. Three to five days old sterile seedlings can be planted in the 21 mm x 5 mm opening at the top of the chamber. A slide is secured on the back of the device (Fig S1D) and a coverslip on the front (Fig S1B) to create an internal cavity with a volume of ~3 ml. The internal grid allows for monitoring of specific areas over time. We filled the chamber with a semi-solid (0.4% Gelzan) Fahraeus medium (33) that supports both plant growth and bacterial motility while remaining transparent for microscope observations. We have tried various inoculation methods and found that mixing a cell suspension at ~10^7 cell/ml in the molten cooled Gelzan prior to addition to the assembled slide-in-chamber followed by introduction of the plant provided the most reproducible results. Under these conditions, the earliest observations that ensure bacterial motility under the conditions of our experiments were at about 4-5 hours post inoculation. The microscope slide-in-chamber can be stored in a humidified vessel and plant-microbe interactions can be observed repeatedly. Here, we utilized confocal microscopy to monitor GFP-expressing *A. brasilense* behavior in real time in the vicinity of the roots over several days.

Chemotaxis guides *A. brasilense* to accumulate and colonize distinct wheat root surfaces

Previous work established that *A. brasilense* colonizes wheat seedling roots (29), so we sought to monitor free swimming behavior and subsequent root surface colonization in physiologically distinct root zones of wheat seedlings: the maturation (root hair), elongation, and root tip zones. We observed that wild type (WT) *A. brasilense* preferentially collected around wheat roots within 5 hours post inoculation (hpi) when compared to a zone away from the root (Fig S2). Cell numbers around the root increase over time (Fig 1A, 2). At 24 hpi, WT accumulated in tight bands within 150 µm of the root surface in the root hair and elongation

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zones (Fig 2A, brackets). Within these bands, cells swam in long runs, with less frequent changes in direction, but on the edges or outside of the bands, cells frequently reversed and swam in shorter runs (Supplemental Movie S1). WT did not accumulate in the root tip zone and the cell numbers in these regions remained low and did not change at all observed time points (Fig 1-2). The increased accumulation of motile cells in specific zones and the formation of tight bands of motile cells that exhibit distinct swimming patterns suggested a role for chemotaxis. We tested this hypothesis using a chemotaxis null strain derivative (Δ*che1*Δ*che4).* Cells of the Δ*che1*Δ*che4* strain are fully motile but are unable to perform chemotaxis (23, 24). We found this strain did not display any accumulation in any root zones observed and it did not form any detectable band of highly motile cells (Fig 2B, D). In fact, cell distribution near the roots was low regardless of the areas of the roots considered. Therefore, a non-chemotactic mutant strain does not preferentially accumulate in any region around the wheat roots, suggesting that chemotaxis mediates the accumulation of motile bacteria near wheat roots.

At 48 hpi, the majority of WT cells were found as non-motile cells on the root surface in the root hair and elongation zones, and fewer free-swimming cells were observed near the roots, suggesting that accumulated cells transitioned to root surface attachment. In contrast, there was no measurable colonization at the root tip (Fig 1A). Non-chemotactic cells did not colonize any root zone. Thus, *A. brasilense* uses chemotaxis to accumulate around root regions that are suitable for subsequent colonization.

A. brasilense chemotaxis produces a different response pattern in the alfalfa rhizosphere

Next, we monitored *A. brasilense* accumulation and colonization of *Medicago sativa* (alfalfa) roots, another plant that *A. brasilense* is known to colonize (15) in the slide-in chamber.

WT accumulated in the root hair and elongation zones of alfalfa, but it did not accumulate at the root tip (Fig 3A,C). Chemotaxis-null cells (Δ*che1*Δ*che4*) did not exhibit preferential accumulation in any specific root zone of alfalfa (Fig 3B), indicating that the response is chemotaxis-dependent. However, the bands of WT motile cells formed in the rhizosphere of alfalfa did not move closer to the root surface over time during the observation period. Furthermore, measurable colonization of alfalfa roots was not observed until 1-week post inoculation, which is delayed compared with *A. brasilense* colonization of wheat, which is observed at 48 hpi (compare Fig 2 to Fig 3C-D). When placed in competition, WT was able to colonize alfalfa roots, whereas the Δ*che1*Δ*che4* mutant strain was not recovered from alfalfa roots (Fig 3D). This result indicates that the mutant was outcompeted by the chemotaxis-competent wild-type strain, confirming the role of chemotaxis in root surface colonization of alfalfa.

Discrete chemotaxis responses in wheat rhizosphere regions suggest the existence of distinct chemical gradients

Next, we used a modified spatial gradient assay (the root-in-pool assay; see Methods section) to monitor bacterial chemotaxis responses upon initial root sensing. In this assay, a root from a seedling which is germinated aseptically, is placed in a pool of motile bacteria and the chemotactic response and accumulation of the bacteria are monitored for up to 15 minutes. In contrast to the slide-in chamber, the root-in-pool allows for monitoring of locomotor behavior immediately after exposure of the bacteria to the root, without having to wait hours for the bacteria to encounter the gradient.

Motile wild-type bacteria accumulated in reproducible patterns within seconds of exposing them to wheat roots in this assay. In the root hair zone, WT cells gathered in a tight band within 200 µm of the root hair zone, and within a brief time (~90 seconds) this band of motile cells moved closer to the root surface (Fig 4A and 4C). Conversely, at the root tip, the WT bacteria formed a band at 100 ± 50 µm away from the tip, with a clearing zone apparent between the root tips and the accumulated cells. The band remained static over at least 15 minutes observation (i.e. it did not move closer to the root surfaces). This band also attracted an increasing number of bacteria, as seen by the broadening of the band (Fig 4A,C). This behavioral pattern suggested cells responded by chemotaxis by moving away from repellent(s). The Δ*che1*Δ*che4* strain did not exhibit this banding pattern or accumulation in any region of the wheat roots, including the tip (Fig 4B) and instead remained as a homogenous pool of cells. These observations indicate that accumulation of motile cells in all three zones of the roots is dependent on chemotaxis and includes both attractant (to root hair and elongation zones) and repellent (root tip) responses. In the root-in-pool assay with alfalfa, the cells remained motile but did not form any visible band, regardless of the zone of the roots observed for the duration of the experiment (Fig 4D). The accumulation of cells as bands around alfalfa roots seen in the slide-in-chamber (Fig 3A) is thus considerably slower since the bands were observed over several hours post-inoculation but a similar accumulation was not detected in the root-in-pool assay that spans minutes rather than hours. Together, these data suggest that motile *A. brasilense* are chemotactically attracted to conditions in the root hair and elongation zones of wheat, which would promote movement of *A. brasilense* closer to these root surfaces over time and root surface colonization. In contrast, motile *A. brasilense* are chemotactically repelled by conditions in the root tip zone, to isolate them from this region, preventing surface colonization.

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One possible explanation for this difference in cell accumulation near wheat and alfalfa roots is the composition of chemicals exuded by the roots. Wheat roots exude compounds that are known to act as strong attractants for *A. brasilense* (34), and we hypothesized that these compounds are either not represented in the exudates of alfalfa or are present but other chemicals may mask the chemotactic response to the attractants. If the composition of root exudates is contributing to bacterial accumulation around roots, we would expect to see a chemotactic response to exudates alone, and we indeed observed this in a chemical-in-plug assay. WT *A. brasilense* exposed to plugs containing wheat exudates formed a tight band close to the plug, and this accumulation was dependent upon functional chemotaxis machinery (Fig S3), while exposure to a plug containing alfalfa exudates did not cause any pattern in *A. brasilense* accumulation.

To further test this hypothesis, we compared the organic compounds found in bulk exudates of wheat or alfalfa roots by mass spectrometry. This analysis identified a total of 121 metabolites common to both wheat and alfalfa root exudates (Fig S4. Our analysis identified organic acids and amino acids as the major metabolites representing 75 (30 amino acid and 45 organic acids derivatives) out of 121 identified metabolites. The remaining metabolites are sugars, purine and pyrimidine metabolism intermediates, and vitamin B6 derivatives. Principal component analysis (PCA) of wheat's and alfalfa's total exudates abundances revealed that the exudate abundance profiles of alfalfa and wheat are mostly non-overlapping (Fig 5A). We found organic acids and amino acids to be broadly represented in the exudates of both wheat and alfalfa roots, but their distribution in each plant root exudate sample was different. Specifically, PCA analysis indicated that while the organic acids abundance profiles of wheat and alfalfa were unique (Fig 5B, Fig. S4), the amino acid abundance profiles of wheat and alfalfa were

overlapping (Fig 5C). These analyses suggest that the organic acids, not the amino acid, profiles explain the divergence between the total exudates of wheat and alfalfa roots, and thus, could be contributing to differences in *A. brasilense* chemotactic behavior in the wheat and alfalfa rhizospheres. Given that *A. brasilense* is preferentially attracted to organic acids, while amino acids are weak attractants (34, 35, 36, 37), we hypothesized that wheat root exudates would have higher abundances of an organic acid(s) that could be acting as an attractant to recruit *A. brasilense*. However, when looking at relative abundance of organic acids, alfalfa exudates had a higher abundances of several organic acids compared to wheat exudates (Fig. S4). The contribution of the organic acids profiles in root exudates to the *A. brasilense* chemotaxis in the wheat versus alfalfa rhizospheres is thus not straightforward. *A. brasilense* utilizes organic acids as preferred carbon sources, while amino acids are poor carbon sources (34, 35, 36, 37) making it possible that metabolism of these compounds could differ in the context of wheat versus alfalfa exudates. In support of this hypothesis, we found that organic acids were more readily depleted in wheat root exudates compared to alfalfa exudates in presence of bacteria, while amino acids were depleted from both root exudates with somewhat similar patterns. Alternatively, the observed changes in abundance of these chemicals in presence of cells may result from changes in exudation patterns of organic acids and to a lesser extent, of amino acids. Together, these results suggest that the role of organic acids in the different chemotaxis response of *A. brasilense* in the wheat versus the alfalfa rhizosphere is likely more complex and could include differences in local gradients of these compounds, presence of other compounds that may modulate chemotaxis responses and/or metabolism in the rhizosphere or a combination of these factors.

A chemoreceptor, Tlp1, mediates attractant responses to organic acids and wheat root hair and elongation zones

Results above indicate that *A. brasilense* chemotaxis mediates the accumulation of motile cells around wheat roots that precedes surface colonization. Previous work has shown that a chemoreceptor, Tlp1, is essential for wheat root colonization (29), prompting us to analyze the chemotaxis behavior of an *A. brasilense* Δ*tlp1* mutant derivative in the slide-in chamber assay. We found that the *A. brasilense* Δ*tlp1* mutant derivative did not accumulate in the root hair and elongation zones (Fig 6, Fig 2), but it accumulated in the root tip region, even forming distinct bands of cells close to the surface of the root tip (Fig 6A). The lack of Tlp1 thus rendered cells unable to detect attractant signal(s) in the root hair and elongation zones while also increasing attraction of the cells in the root tip regions. We used the root-in-pool assay to further test these assumptions and found that the Δ*tlp1* mutant derivative behaved similarly in the short-term assay as in the longer-term slide-in chamber assay. In this assay, Δ*tlp1* cells formed a band close to the root tip and displayed weak (seen as a very faint band of motile cells) accumulation in the root hair zone (Fig 6B). There was no detectable accumulation of cells in the elongation zone. The Δ*tlp1* mutant was also impaired in colonizing corresponding regions of the root surfaces as the bacterial density in the root hair and elongation zones showed large variations that suggested that cells were impaired in the ability to permanently colonize these regions. On the other hand, cell density increased at the surface of the root tips, in contrast to the wild type colonization pattern (Fig 2, 6).

Previous work has shown that Tlp1 mediates attractant chemotaxis response to organic acids and contributes to the repellent response of *A. brasilense* away from redox active compounds, with the mechanism of this dual effect yet to be elucidated (29). We hypothesized that Tlp1 may sense attractant gradients of organic acids originating from the root hair and/or elongation zones. We confirmed the role of Tlp1 in sensing organic acids using a chemical-in-

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plug assay (Fig S5). WT cells exhibited attractant responses to spatial gradients of malate, pyruvate, and succinate, and this response was absent in a motile but non-chemotactic mutant strain (Fig S5). Cells lacking Tlp1 displayed reduced attractant responses to spatial gradients of pyruvate and succinate and no response to gradients of malate, suggesting malate is the major attractant sensed by Tlp1. Organic acids are well represented in wheat root exudates, including organic acids such as malate (Fig S4). In presence of *A. brasilense*, malate, as well as other organic acids, was also rapidly depleted from wheat roots exudates (Fig S4). These results are consistent with Tlp1 mediating chemotaxis to metabolizable attractants, such as malate, exuded by wheat roots. These data further suggest that organic acids are major components of root hair and/or elongation zones exudates that attract *A. brasilense*.

Repellent responses to wheat root tips and ROS are mediated through c-di-GMP and C-terminal PilZ domains of chemoreceptors

Cells lacking Tlp1 are no longer repelled from the wheat root tip, and instead accumulated close to the wheat root tip surface. No obvious repellents were identified in the exudates analyzed (Fig S4). Plant root tips produce reactive oxygen species (ROS) (38) which could act as redox active repellents for *A. brasilense*. The chemotaxis response of *A. brasilense* to ROS is not known. We thus tested chemotaxis in gradients of ROS compounds by exposing motile cells inoculated into a soft agar plate to spatial gradients of different ROS generating compounds. We tested bacterial behavioral responses to spatial gradients of hydrogen peroxide and cumene peroxide or buffer added to paper discs and incubated the plates at room temperature for 20 minutes before measuring the resulting motility responses. WT cells moved 0.44 ± 0.05 cm away (as seen with the clearing zones around the discs) from hydrogen peroxide saturated

discs within 20 minutes, and this response depended on functional chemotaxis (Fig 7A). Migration away from the spatial gradient of ROS was specific to hydrogen peroxide, as none of the strains tested displayed any visible motility responses to spatial gradients of cumene peroxide (Fig 7A). *A. brasilense* thus responds to repellent gradients generated by hydrogen peroxide. The Δ*tlp1 cells* did not respond to hydrogen peroxide gradients but the repellent response was restored by expressing Tlp1 *in trans* in the Δ*tlp1* mutant strain background (Fig 7A). Despite differences in chemotaxis responses, both WT and Δ*tlp1* strains had similar susceptibility to killing by hydrogen peroxide with 3% hydrogen peroxide killing WT (9.9±1.4 mm zone of inhibition) and Δ*tlp1* (9.9 ±2.2 mm zone of inhibition) strains. Therefore, *A. brasilense* responds chemotactically to gradients of hydrogen peroxide and Tlp1 mediates these repellent responses. We hypothesize that the *A. brasilense* repellent responses seen at the wheat root tips could be mediated by repellent signals such as hydrogen peroxide that are detected by Tlp1.

The results above prompted us to probe the mechanism(s) by which Tlp1 could sense effectors as attractants (organic acids) and repellents (hydrogen peroxide). Like Tlp1, Aer is important for chemotaxis to wheat roots including the repellent response to the root tips (31) and like Tlp1, Aer possesses a C-terminal PilZ domain. Since Aer and Tlp1 have unrelated ligand binding domains (29, 31), we hypothesized that the repellent response to the root tips depended on the presence of the PilZ domain. We thus characterized the role of *A. brasilense* Aer in the chemotaxis response away from spatial gradients of hydrogen peroxide. Like the Δ*tlp1* mutant, the Δ*aer* failed to chemotactically respond to spatial gradients of hydrogen peroxide in the ROS disk assay and the repellent response was restored by expressing a parental Aer (Fig 7B). Thus, the repellent responses to hydrogen peroxide mediated by Tlp1 and Aer appear to depend on the presence of a PilZ domain, regardless of the ligand binding domain. We next determined the

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chemotaxis responses of Δ*tlp1* or Δ*aer* mutants, each expressing variants of Tlp1 and Aer, unable to bind c-di-GMP, i.e. (Tlp1$^{R562A\ R563A}$ (39) or AerΔPilZ (31)). Surprisingly, while cells expressing these variants were repelled away from hydrogen peroxide, strains expressing Tlp1$^{R562A\ R563A}$ did not migrate as far as WT *A. brasilense* (0.38 ± 0.08 cm) and the AerΔPilZ expressing strain had great variability in distance migrated (0.46 ± 0.15 cm) and the strength of the response (rings were frequently fainter than a WT response). The responses of both mutants were compromised since the cells consistently produced "blebs" at variable distance from the discs (Fig 7A, B), suggesting the response to the hydrogen peroxide gradient is only partially restored and/or that the cell population is heterogenous in the level of expression of the constructs. Furthermore, both of these mutants had weak responses as rings were not as evident as WT.

PilZ domains bind c-di-GMP (40-42). To clarify the potential role of c-di-GMP metabolism in mediating repellent responses to ROS, we used an optogenetic plasmid system that permits the transient manipulation of c-di-GMP intracellular levels on a timescale consistent with chemotaxis (43). This system consists of a plasmid constitutively expressing a red-light activated diguanylate cyclase and plasmid expressing a blue-light activated phosphodiesterase (43, 44). Our previous work showed that illuminating cells carrying these plasmids expressing these proteins with red or blue light for 10 sec is sufficient to activate their catalytic activity and to increase (red light) or decrease (blue light) intracellular c-di-GMP levels without affecting motility. The increase/decrease in c-di-GMP level is transient, lasting less than 10 min (43, 44). Lowering c-di-GMP levels in WT cells using this optogenetic system rendered cells unable to respond to spatial gradients of hydrogen peroxide (Fig 7C), while cells experiencing increased c-di-GMP levels responded to spatial gradients of hydrogen peroxide 10 min sooner than the wild

type, regardless of the concentrations of hydrogen peroxide (Fig 7D). These data thus implicate changes in intracellular c-di-GMP levels in the repellent response of *A. brasilense* to spatial gradients of hydrogen peroxide, most likely via binding to the PilZ domain of chemoreceptors such as Tlp1 and Aer.

Chemoreceptor PilZ domains control attractant chemotaxis to rhizosphere gradients

We have previously obtained evidence that the C-terminal PilZ domain of Aer may function to integrate signaling with other, unknown chemoreceptors with which Aer clusters (31). One of the striking observations supporting this hypothesis is that expressing an AerΔPilZ variant in the Δ*aer* background not only failed to rescue discrete attractant and repellent responses to distinct wheat root regions but it also abolished the ability of cells to navigate gradients originating from roots, but it also produced additional detrimental effects that suggested complete lack of chemotaxis signaling, resulting in these cells behaving similarly to a non-chemotactic strain (31). Given their related signaling domain topology and the presence of C-terminal PilZ domains, Aer and Tlp1 may function in the same chemotaxis signaling cluster. If this hypothesis is correct, we would expect that expressing a variant of Tlp1 with a defective PilZ domain ($Tlp1^{R562A\ R563A}$) would produce chemotaxis defects similar to those we have previously observed with the Δ*aer* strain expressing AerΔPilZ. To test this hypothesis, we analyzed the behavior of the *A. brasilense* Δ*tlp1* mutant strain expressing either a parental Tlp1 or a variant unable to bind to c-di-GMP ($Tlp1^{R562A\ R563A}$)(39) in the root-in-pool assay (Fig 6C). While expressing a parental Tlp1 restored the wild type behavior, expressing the $Tlp1^{R562AR563A}$ variant did not. In addition, this non-c-di-GMP binding Tlp1 variant had a more deleterious effect on chemotaxis near wheat roots than that observed for the mutant strain lacking Tlp1

alone: cells did not respond to any region of the roots and behaved like a non-chemotactic mutant strain while the Δ*tlp1* strain still responded to the root tips (Fig 6C). Thus, a functional PilZ domain of Tlp1 is required for *A. brasilense* to sense complex gradients generated by wheat roots in this assay. The similar additive effects that expressing Tlp1 or Aer variants with nonfunctional PilZ domains have on chemotaxis signaling further support the hypothesis these chemoreceptors function in the same chemotaxis signaling cluster array.

Discussion

Here, we connect patterns of chemotaxis responses observed in real time to chemical gradients that distinguish different plants as well as different regions of the roots to potential chemoeffectors present in exudates and sensed by dedicated chemoreceptors. We also identify a critical role for second messenger signaling by c-di-GMP in modulating these responses. Together, the tools and approaches used here allow bacterial behavioral responses and root surface colonization patterns to be tracked at relevant temporal and spatial scales and are applicable to diverse plants and root-associated motile chemotactic bacteria.

We show that bacterial chemotaxis modulates the root colonization patterns of *A. brasilense* to different plants. Chemotaxis-dependent accumulation of *A. brasilense* in specific regions of the roots determined the ability to colonize the corresponding root surfaces. While chemotaxis was required for competitive root surface colonization of both wheat and alfalfa, our results indicate that the ability of *A. brasilense* to metabolize attractants, in particular organic acids, found in the rhizosphere of host plants mediates the ability of the cells to quickly accumulate as bands near the roots by chemotaxis and to subsequently colonize the root surfaces. Interestingly, while organic acids were detected in the root exudates of wheat and alfalfa, they

were significantly depleted when wheat exudates, but not alfalfa, were exposed to *A. brasilense*. Given the short exposure time of bacteria to the exudates, the known metabolic capacity of *A. brasilense*, their subsequent behavioral responses and our bulk analysis of root exudates composition, we surmise that depletion of specific compounds from exudates resulted from bacterial metabolism of major compounds, although we cannot exclude the possibility that exposure to bacteria triggered changes in the root exudation pattern of the plants. Since PCA analysis revealed divergence in organic acid exudation between wheat and alfalfa, it follows that metabolism-dependent chemotaxis, including toward organic acids, is likely to drive the behavior of *A. brasilense* in the wheat rhizosphere while it would play a minor role in the alfalfa rhizosphere. Most chemotaxis responses in *A. brasilense* are metabolism-dependent and described as energy-taxis, i.e. the bacteria chemotax in response to effectors that directly alter energy metabolism: strong attractants are organic acids that are easily metabolized and increase intracellular energy while repellents are compounds that affect energy-generating processes such as oxidized quinones (34). Metabolizing attractants found in root exudates would ensure the corresponding chemical gradients are shallow which in turn, would sustain strong behavioral responses. In addition to energy taxis, it is possible that metabolizing root exudates compounds could alter the chemoreceptor repertoire of *A. brasilense*. The strength of a chemotaxis response toward an individual compound largely depends on the affinity of the chemoreceptor for this compound as well as the chemoreceptor abundance (45). In *Pseudomonas putida*, the abundance of chemoreceptor transcripts changes with growth conditions and in the presence of maize root exudates (46, 47). Previous work in our laboratory has demonstrated that the abundance and contribution of at least one of the 51 chemoreceptors encoded in the genome of *A. brasilense* to the chemotaxis response changed with combined nitrogen availability to promote navigation of

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420 air gradients compatible with nitrogen fixation (30). It is thus possible that root exudates
421 modulate the abundance of at least some chemoreceptors to promote cell accumulation in the
422 vicinity of roots, although this remains to be experimentally tested.

423 The results obtained here also identify c-di-GMP as a major regulator of PilZ-domain
424 containing chemoreceptors signaling activity in the vicinity of wheat roots. Δ*tlp1* or Δ*aer* strains
425 expressing Tlp1 or Aer variants unable to bind c-di-GMP lacked attractant and repellent
426 responses in the wheat rhizosphere, although previous work has showed that these strains
427 displayed chemotaxis responses to gradients of a single chemoeffector such as oxygen or malate
428 (31, 39, 43, 48). Similarly, functional PilZ domains, able to bind c-di-GMP, were required for
429 the repellent response to hydrogen peroxide mediated by Tlp1 and Aer. These observations
430 suggest that functional PilZ domains and c-di-GMP binding to these domains on chemoreceptors
431 are necessary for recognizing and integrating cues from complex gradients found in the vicinity
432 of plant roots. How could c-di-GMP binding to one chemoreceptor modulate chemotaxis to
433 complex cues such as those found in the wheat rhizosphere while permitting chemotaxis to
434 gradients of a single chemoeffector? One possibility may lie in the organization of
435 chemoreceptors in large ordered arrays that are clustered at the cell poles (49). *A. brasilense*
436 possesses two spatially distinct membrane-bound chemotaxis arrays at the cell poles with
437 chemoreceptors segregating into arrays based on heptad repeat length (36H and 38H) (50, 51).
438 The genome of *A. brasilense* encodes 6 PilZ domain-containing chemoreceptors which all
439 belong to the 38H length class and are thus expected to be clustered together into a single array,
440 together with other 38H chemoreceptors and spatially segregated from the 36H chemotaxis
441 signaling array (50). In chemotaxis signaling arrays, chemoreceptors are allosterically coupled
442 (52). With such spatial arrangement, the deletion of a single PilZ-domain containing

chemoreceptor, such as in the Δ*tlp1* and the Δ*aer* strains, would not perturb the signaling ability of the chemotaxis signaling array, although it would likely affect chemotaxis response sensitivity. Consistent with this, we observed changes in the sensitivity of variants lacking functional PilZ to oxygen gradients (31). We hypothesize that in the absence of c-di-GMP, the PilZ-containing chemoreceptors would adopt a conformation that abolishes signaling competence by the chemotaxis array. Given that the majority of *A. brasilense* chemoreceptors belong to the 38H class (50), it is likely that such a conformational change would alter chemoreceptor packing in such a way as to render cells unable to integrate and respond to most chemical cue gradients. Our previous observations that lack of c-di-GMP binding to Tlp1 (39, 43) and Aer (31) appears to inhibit their ability to signal during chemotaxis are consistent with this possibility. Furthermore, PilZ domains are known to undergo large conformation changes when binding to c-di-GMP (40, 53-55). Given the allosteric packing and interaction of chemoreceptors in chemotaxis signaling arrays, it is conceivable that binding of c-di-GMP to PilZ-domain chemoreceptors could influence receptor signaling state, as well as that of allosterically coupled neighboring chemoreceptors. This hypothesis predicts that cells should be able to process some cues through the 36H chemoreceptor array, which would lack PilZ domain-containing chemoreceptors, although the sensing versatility of 36H cluster-associated chemoreceptors is expected to be significantly limited given that only three 36H chemoreceptors are predicted to comprise this cluster (50). Together these findings support a model in which the two chemotaxis arrays found in *A. brasilense* are functionally distinct, with one of the two chemotaxis signaling arrays integrating c-di-GMP metabolism with chemotaxis in this species. Our previous work has shown that c-di-GMP levels change with ambient oxygen levels and with carbon sources available for growth (39, 43). Changes in c-di-GMP levels with metabolism may

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466 thus contribute to *A. brasilense* metabolism-dependent chemotaxis responses. The only
467 previously reported repellents for *A. brasilense* were redox active compounds such as quinones
468 or inhibitors of electron transport chain that were thought to directly interfere with energy-
469 sensing chemoreceptors (34). Here we identify hydrogen peroxide as a repellent for *A.*
470 *brasilense*. While redox active compounds may directly alter the signaling activity of
471 chemoreceptors that possess redox motifs such as FAD in their sensing domains as present in
472 Aer or AerC (30, 31), our results here suggest that the repellent effect of hydrogen peroxide on
473 *A. brasilense* chemotaxis may be mediated through changes in c-di-GMP levels that would alter
474 the conformation and thus signaling activity of PilZ-containing chemoreceptors. These results
475 thus also support a role for c-di-GMP in coupling metabolism with chemotaxis in *A. brasilense*.
476 ROS are critical to plant growth and root tips are actively growing regions that are known
477 for producing ROS (56, 57). Our results show that *A. brasilense* is chemotactically repelled by
478 hydrogen peroxide, which could be produced by wheat root tips. Our results do not establish that
479 *A. brasilense* senses root-generated hydrogen peroxide, but they do support a model in which
480 motile *A. brasilense* use chemotaxis to avoid accumulating in root zones likely to generate toxic
481 ROS. Previous work has shown that inoculation of *A. brasilense* to wheat root seedlings
482 modulate the production of ROS species such as superoxide and the activity of plant enzymes
483 that mitigate ROS stress (38). Furthermore, successful colonization of plant roots by *A.*
484 *brasilense* depends on the ability of the bacteria to overcome oxidative stress (58). Together
485 these data suggest that the ability to sense and respond to ROS species is critical for the
486 association of *A. brasilense* with plant roots. *Helicobacter pylori* is also able to sense host-
487 generated ROS, including hydrogen peroxide, by chemotaxis and this response is required for the
488 ability of the bacterium to colonize the gastric epithelial glands (59). In *H. pylori*, sensing of

oxidative stress and ROS is mediated by the C-terminal CZB domain of the cytoplasmic TlpD receptor (60). The C-terminal CZB domain binds zinc (61) with homologs found at the C-terminus of chemoreceptors of bacterial pathogens that colonize mucosal surfaces, suggesting its role in sensing ROS may be widespread (59). Interestingly, TlpD also forms an autonomous chemotaxis signaling cluster that mediates chemotaxis responses to oxidative stress (60). These observations suggest that the presence of the C-terminal CZB domain, like the PilZ domain, may confer unique conformational constraints that may be incompatible with clustering with other chemoreceptors. These results raise the possibility that the C-terminal CZB domain of TlpD, as well as other C-terminal domains identified in chemoreceptors (36), functions to couple chemotaxis signaling to metabolism via detection of small molecules/metals.

Organic acids, such as malate, are represented in wheat root exudates and consumed by *A. brasilense* with evidence suggesting that Tlp1 senses these organic acids as attractants. While we have not determined the exact mode of detection of organic acids by Tlp1, a diverse class of unrelated ligand binding domains from a broad range of chemoreceptors were shown to bind diverse organic acids and these are well represented in the genome of soil bacteria (62). Chemotaxis to organic acids has also been implicated in the association of diverse bacteria with the roots of plants (13, 63, 64). The work here thus identifies the root elongation and root hair zones as major areas where such organic acids are exuded to attract bacteria. Data obtained here imply that as motile *A. brasilense* cells approach roots of plants, they experience gradients due to root exudates which not only change their metabolism through consumption of primary metabolites but also affect signaling, including through c-di-GMP. The dynamic integration of these events ultimately shapes the behavior and physiology of motile bacteria in the rhizosphere and determines their ability to colonize the root surfaces.

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Materials and Methods

Bacterial growth and maintenance

All strains used throughout this study are detailed in Table 1. All *A. brasilense* strains were maintained on 1.5 % agar minimal media for *A. brasilense* (MMAB) containing 10mM malate as a carbon source (65) or 1.5 % agar Tryptone Yeast media (TY) with appropriate antibiotics: ampicillin (200 µg/mL), kanamycin (30 µg/mL), and/ or tetracycline (10 µg/mL). For liquid cultures, all *A. brasilense* strains were grown shaking (180 rpm) in MMAB with 10 mM malate as a carbon source and 20 mM ammonium chloride as a nitrogen source with appropriate antibiotics.

Generation of GFP expressing *A. brasilense*

To generate constitutively fluorescent strains, we used the broad host range vector pHR-GFPTc (Table 1) (63). pHR-GFPTc was transferred into Sp7 (WT), Δ*tlp1* and Δ*che1*Δ*che4* strains using biparental conjugation as previously described in (62). Transconjugants were selected for on MMAB containing tetracycline and confirmed using a Nikon ECLIPSE 80i fluorescence microscope equipped with a Nikon CoolSnap HQ2 cooled CCD camera.

Germination of *T. aestivum* and *M. sativa*

T. aestivum (wheat) and *M. sativa* (alfalfa) were utilized throughout this study. *T. aestivum* were sterilized with successive washes of bleach, 70% ethanol containing 1% Triton X-100, and sterile water. After sterilization, seeds were planted into 0.3% agar and placed in the dark for 48 hours to germinate. Then, plates were placed in the light and allowed to grow for 24 hours. All assays were performed on germinated seedlings 3-5 days old.

Slide-in-chamber dimensions and assembly

The microscope slide-in chamber (85 mm x 35 mm x 8 mm) was designed in Autodesk Inventor and printed from ABS thermoplastic filament with a Fortus 250MC 3D printer (https://www.kerafast.com/product/2029/slide-in-plant-chamber). The print layer height was set to 0.007 inches (32). Microscope slides and coverslips were attached to the slide-in chamber using E600 industrial strength adhesive. A string wick was threaded through the 0.4 cm hole in the bottom of the chamber to hydrate the chamber over the course of a week.

Cells were grown to late log phase, washed with Che buffer and suspended in Che buffer and mixed by repeated inversion in a 1:1 ratio with molten Fahraeus semi-solid media (33)($CaCl_2$, 100 mg l^{-1} , $MgSO_4 \cdot 7H_20$, 120 mg l^{-1}, KH_2PO_4, 100 mg l^{-1}, Na_2HPO_4, 150 mg l^{-1} and ferric citrate 5 mg l^{-1} 0.3% agar). Molten Fahraeus was used to plug the bottom of the chamber and then the bacteria-Fahraeus mixture was used to fill the interior of the chamber. Germinated seedlings were planted at the top opening with roots directed downward. Chambers were allowed to solidify upright for several hours before imaging. Chambers were stored in humidified vessels and imaged for up to 1-week post inoculation. Between 5 and 10 chambers were observed for each inoculation condition.

Root-in-pool assay

Five ml of cells were grown to O.D.600 =0.4 in MMAB containing malate as a carbon source. After growing, cells were washed 3 times with Che buffer (1.7 g L^{-1} dipotassium phosphate, 1.36 g L^{-1} monopotassium phosphate; 0.1 mM EDTA) and re-suspended in 5 ml Che buffer. A black washer with a slit cut in it was secured to a depression slide (VWR) using vacuum grease. The germinated root was placed in the slit and 400 µL of washed bacteria was used to fill the chamber created by the dip and washer, and then a cover slip was used to cover the pool chamber (31).

All root-in-pool assays were recorded using the 4x objective of a Nikon E200 phase contrast microscope equipped with Nikon Coolpix digital camera. Assays were observed for 15 minutes and the first 3 minutes was filmed using the Nikon Coolpix digital camera. FIJI was used for all image and video analysis (67). All root-in-pool assay videos were converted to 8-bit and background subtraction was performed and the Gaussian blur filter was applied. Intensity (bacterial accumulation) was measured along a straight line out from the root zone of interest every 30 seconds and plotted. The root-in-pool assays were repeated between 3 and 5 times for each strain analyzed.

Fluorescent microscopy and image analysis

All chambers were imaged using a Zeiss LSM710 confocal microscope. Images were analyzed using FIJI (67). All distance measurements were obtained using the measure tool. Calculated total fluorescence was measured by splitting the channels and using the Time Series analyzer plug in. The root surface was identified through the z-plane series, and an area of interest was selected. The area, mean gray value, and integrated density were measured on both the red and green channels of the area of interest. Calculated Total Corrected Fluorescence (CTCF) for each channel was calculated using: Integrated Density—(Area of selection x Mean Fluorescence of Background). Bacterial density and colonization was determined by dividing CTCF of the GFP signal by CTCF of the root autofluorescence to account for signal overlap. Student's t-tests were performed to determine if colonization levels were significantly different between WT, Δ*tlp1* and Δ*che1*Δ*che4*. A p-value < 0.05 was used to determine significance.

Long-term colonization

Long term colonization (5-day) was determined using a previously described assay (23). Cells were grown to OD600= 0.5 and 2mL of the culture was washed with Che buffer (1.7 g L^{-1}

dipotassium phosphate, 1.36 g L^{-1} monopotassium phosphate; 0.1 mM EDTA) and concentrated in 400 µL of Che buffer. A 6.5 cm diameter growth container was filled with 50 ml semi-solid Fahraeus and allowed to solidify. Four plants (germinated, as described above) were placed in the media at the edges of the chamber. Twenty microliters of the washed and concentrated bacteria were inoculated into the center of the chamber. To determine the amount of bacteria inoculated into the chamber (input), serial dilutions and CFU counts were performed on this initial inoculum. Growth chambers were incubated at 25°C for 5 days. After 5 days, roots from all 4 plants were combined, massed, homogenized in 400 µL Che buffer. Samples were also taken from the periphery of the chamber away from the bacterial inoculation point or plants. Samples were serially diluted and CFU counts were performed to determine colonization levels (recovered). All CFU counts were grown at 28°C for two days and then counted. Colonization efficiency was quantified by: $\log(CFU_{recovered})/\log(CFU_{input})$.

Root exudate collection and analysis

Wheat seeds were sterilized and germinated as described above. Seedlings were separated into sterile 12-well plates with 2 ml sterile water per well (2 plantlets/ well). Seedlings were incubated for 24 hours in the plates in the dark, and the water was collected, filter sterilized using a 0.45 micrometer filter, and lyophilized to collect and concentrate exudates. Seedlings were dried and weighed. For seedlings treated with *A. brasilense,* exudates were collected in the same manner after 24 hours. After 24 hours, wild type *A. brasilense* was inoculated into each well of the plate and allowed to incubate for 30 minutes at room temperature. Before inoculation, wild type (Sp7) *A. brasilense* was grown to exponential phase (O.D.600= 0.4-0.8) and cells were collected, washed with sterile water, and concentrated to O.D.600=1, and 500 microliters of concentrated cells was used for inoculum for each well. After collection, exudates were filter-

sterilized using 0.45 micrometer filters and lyophilized and sent to the Biological and Small Molecular Mass Spectrometry Core at the University of Tennessee (https://chem.utk.edu/facilities/biological-and-small-molecule-mass-spectrometry-core-bsmmsc/). The lyophilized exudates were resuspended in 1 ml of water and separated by Hydro RP HPLC. Samples were ionized by electrospray in negative mode on an Orbitrap Mass spectrometer. Data was processed and peaks picked via Maven software. Metabolite area counts were normalized to dry sample weight. The heat map for relative abundance was generated by taking the log10 of the normalized area for each of 3 biological replicates and averaging. Fold change was calculated by dividing the average abundance of each compound under various conditions and then log2 transforming. Principal component analysis (PCA) on the log10 of exudate abundances of wheat and alfalfa triplicates was performed using a customized Python algorithm with the scikit-learn package (68).

Chemical-in-plug assay

To generate chemical in plugs malate (10mM), pyruvate (10mM), or succinate (10mM) in sterile water were mixed with molten 1% low melting point agarose in sterile water (Thermo-Fisher). For plugs containing exudates, exudates were extracted and lyophilized as described above. Exudates were resuspended in 5 ml of sterile water and mixed 1:1 with molten 2% low melting point agarose. Ten microliter plugs were allowed to solidify on a depression slide (VWR), and 150 µl of bacteria was placed on the depression slide and covered with a coverslip. Bacterial behavior was monitored using the 4x objective of a Nikon E200 phase-contrast microscope. Behavior was videoed using a C-mounted Nikon Coolpix digital camera for 5 minutes. Slices were obtained from videos using FIJI.

Spatial gradient assay for ROS chemotaxis

Twenty-five ml of cells were grown to OD600=0.5 in TY liquid with appropriate antibiotics. The entire culture was washed with Che buffer and resuspended in 25 ml of Che buffer and mixed with 25 ml of TY semi solid (0.3% agar) (1:1, v/v). Twenty-five ml of bacteria-semi solid TY mixture were poured into square petri dishes. Sterilized filter paper disks soaked in 20 µl hydrogen peroxide, cumene peroxide, or Che buffer were placed on top of the bacteria-agar mixture and chemotactic response was monitored every 5 min for 2 hours. A response was defined as the formation of a visible ring of bacteria away from the filter paper.

For cells carrying plasmids encoding light activated enzymes (pBlue-PDE or pRED-DGC)(43, 44), cultures were grown to OD600=0.5 in TY liquid with appropriate antibiotics (Table 1) and maintained in the dark, and plates were exposed to red (610 to 730 nm) or blue light (450 nm) for 30 sec using a lamp equipped with a Magic Lighting Light bulb and remote control immediately before exposure filter paper. After exposure, plates were maintained in the dark, and non-inducing green light (505-575 nm) was used for observing response and obtaining images. The images were obtained using a Nikon CoolPix digital camera.

Measuring *A. brasilense* sensitivity to hydrogen peroxide via zone of inhibition assay

WT and Δ*tlp1* were grown in TY liquid culture until OD600=0.8. Five hundred microliters of cells were spread on TY solid with ampicillin. Once the plates dried, sterile filter paper discs were soaked in Che buffer or hydrogen peroxide (3%, 0.3%, or 0.03%) (v/v) (Fisher Scientific) and placed on the plates. After 48 hours of incubation at 28°C, zones of growth inhibition were measured.

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Figure legends

Figure 1: Interaction of WT and non-chemotactic (Δ*che1*Δ*che4*) *A. brasilense* with wheat roots in the slide-in chamber. WT and chemotaxis null (Δ*che1*Δ*che4*) bacteria carrying pHRGFP to constitutively express GFP were mixed with the semi-solid molten medium used to fill the chamber. Seedlings were planted at the top of the chamber (see Materials and Methods for details). Bacteria are visible in green and the wheat roots are red. (A, B) WT and Δ*che1*Δ*che4* accumulation close to the root surface in the root hair and elongation zones. Bands of motile fluorescent bacteria are denoted by brackets and the average bandwidth in micrometers is noted

below the picture. Images are representative of 3 biological replicates in different chambers. (C, D). Scale bars represent 100 micrometers.

Figure 2: Quantification *of A. brasilense* colonization levels on wheat roots. Colonization levels in each root zone were determined by measuring fluorescence of surface attached cells normalized to the area of the root observed and expressed in arbitrary units. Calculated Total Corrected Fluorescence (CTCF) for each channel was calculated using: Integrated Density—(Area of selection x Mean Fluorescence of Background). T-tests were used to determine if WT colonization differed significantly from *Δche1Δche4* colonization. An * indicates a significant difference from the *Δche1Δche4* strain (p value $\leq$ 0.05).

Fig 3: WT and chemotaxis null *(Δche1Δche4*) behavior in the presence of alfalfa roots. Free swimming WT (A) and *(Δche1Δche4*) (B) cells accumulation around the alfalfa roots in the slide-in chamber. Time course colonization of alfalfa roots by WT (C) and Δ*che1*Δ*che4* (D) in the slide-in chamber. *A. brasilense* constitutively expressing GFP colonization was inoculated into a slide in-chamber containing 3-day old wheat and were imaged over the course of 72 hours post inoculation (hpi). *A. brasilense* is visible in green and the wheat roots are red (autofluorescence). Images are representative of 3 chamber replicates. In chamber (C, D) colonization levels were quantified by calculating CTCF (Calculated Total Corrected fluorescence) of the attached bacteria and normalizing to root area. Five-day colonization was measured by CFU counts from homogenized roots (E). NR indicates no bacteria recovered. A t-test indicated no significant difference from *Δche1Δche4* colonization (p-value $\leq$ 0.05) for colonization in any of the root zones.

880

881 **Fig 4:** Interaction of wild type (WT) and non-chemotactic *A. brasilense* (Δ*che1*Δ*che4*) in the
882 root-in-pool assay with intact roots from wheat and alfalfa seedlings. (A and B) Wheat roots
883 from intact seedlings were placed in a suspension of motile WT (A) or chemotaxis null mutant
884 (B) strains and free-swimming behavior in the vicinity of the roots was observed and recorded
885 over time. WT *A. brasilense* accumulates in a band in the root hair and tip zones (indicated by
886 the white arrow), while the chemotaxis null strain forms a homogenous pool that does not change
887 within 10 minutes of observation. (C) Intensity profile analysis of images shown for the WT cell
888 suspension in the root-in-pool assay at 0, 30, and 60 sec. D) WT and chemotaxis null strains in
889 the presence of root tip (left) and root hair zone (right) of alfalfa seedlings in the root-in-pool
890 assay.

891

892 **Fig. 5**: Principal Component Analysis (PCA) of metabolites detected in wheat and alfalfa root
893 exudates. (A) PCA loading plot of log10 abundance of total metabolites isolated from three
894 biological samples of wheat (black) and alfalfa (blue). 95% confidence interval of the PCA
895 scores' covariances from three samples of wheat and alfalfa are represented as ellipses. (B) of
896 only organic acids isolated from three biological samples of wheat (black) and alfalfa (blue).
897 95% confidence intervals of the PCA scores' covariances from 3 samples of wheat and alfalfa
898 are represented as ellipses. (C) Principal component analysis (PCA) loading plot of $\log_{10}$
899 abundances of only amino acids isolated from three biological samples of wheat (black) and
900 alfalfa (blue). 95% confidence intervals of the PCA scores' covariances from 3 samples of wheat
901 and alfalfa are represented as ellipses.

902

903 **Fig 6:** *A. brasilense* Δ*tlp1* mutant strain free-swimming response and colonization of wheat
904 roots. The Δ*tlp1* strain in the slide-in-chamber with wheat seedlings (A). Time course of the
905 colonization of wheat root surfaces by the *A. brasilense* Δ*tlp1* mutant strain (B). The chambers
906 were filled with Fahraeus medium containing *A. brasilense* constitutively expressing GFP
907 (pHRGFP) and were imaged over the course of 72 hours. *A. brasilense* is visible in green and
908 the wheat roots are in red. Images are representative of 3 biological replicates in different
909 chambers. Colonization levels were quantified by calculating Calculated Total Corrected
910 Fluorescence (CTCF) for each channel was calculated using: Integrated Density/(Area of
911 selection x Mean Fluorescence of Background). A t-test indicated no significant difference from
912 WT *A. brasilense* colonization (p-value ≤ 0.05) for colonization in any of the root zones. (C) The
913 role of Tlp1 and c-di-GMP binding to Tlp1 in responding to wheat roots. A root-in-pool assay
914 was used to observe the behavior of cells lacking Tlp1 (Δ*tlp1*) or expressing Tlp1 impaired in
915 binding to c-di-GMP ($Tlp1^{R562A\ R563A}$) in the presence of wheat. Arrows indicate accumulation of
916 motile cells at that position.
917
918 **Fig 7:** The role of PilZ domain chemoreceptors and c-di-GMP level in responding to ROS
919 gradients. Motile *A. brasilense Δtlp1* (A) or *Δaer* (B) were exposed to gradients generated by
920 filter paper soaked in buffer, hydrogen peroxide, or cumene peroxide. To determine the role of
921 intracellular c-di-GMP levels in responding to ROS gradients, c-di-GMP levels were
922 manipulated using an optogenetic plasmid system, as described in the Methods (C-D). WT *A.*
923 *brasilense* cells with a red-light activated diguanylate cyclase (pRED-DGC) or blue-light
924 activated phosphodiesterase (pBLUE-PDE) were illuminated with green (control), red, or blue
925 light before exposure to filter paper soaked in Che buffer or various concentrations of hydrogen

peroxide. A chemotactic response is indicated by a ring forming a certain distance away from the filter paper. Black arrows indicate an accumulation of motile cells. Positive responses are denoted by a black arrowing pointing at the ring formed by bacteria. The plates were observed every 5 minutes.

Supplemental Figure legends

Fig S1: The slide-in chamber for observing plant-microbe interactions in real time. The chamber consists of a grid, wick, coverslip, and slide. Panels A-D show the front (A-B), and back (C-D) of the slide-in-chamber with a coverslip (B) and slide (D). The inside is filled with a semi-solid medium and inoculated with microbes and germinated seedlings are planted at the top. A germinated wheat seedling (E) planted in the semi-solid medium inside the chamber. The chamber allows for the seedling to grow and be monitored for up to a week.

Fig. S2: Behavior of WT and non-chemotactic (*Δche1Δche4*) *A. brasilense* 6-7mm away from wheat roots in the slide-in chamber at 5 hpi. WT and chemotaxis null (*Δche1Δche4*) bacteria carrying pHRGFP to constitutively express GFP were mixed with the semi-solid molten medium used to fill the chamber.

Fig S3: *A. brasilense* response to agarose plugs containing wheat or alfalfa exudates. The formation of a band away from the plug indicates a chemotaxis response. Black arrows indicate the edge of the exudate containing plug. White arrows point to bacterial accumulation.

948 **Fig S4:** Heatmap depicting log_2-transformed fold changes for relative abundances of known
949 metabolites. Red indicates a positive and blue a negative fold change. A significant difference
950 (Student's t-test, p-value $\leq$ 0.05) between wheat and alfalfa exudates are indicated with +, while
951 a significant difference between bacteria treated exudates and non-treated exudates is indicated
952 with *. P-value < 0.05 is indicated by + or *, while p-value< 0.01 is indicated by ++, or **.
953
954 **Fig S5:** *A. brasilense* response to organic acids. *A. brasilense* response to low melting point
955 (LMP) agarose plugs containing 10 mM organic acids as indicated above each image. The LMP
956 only plug contains buffer only. Black arrows indicate the edge of the organic acid containing
957 plug. White arrows point to bacterial accumulation.
958
959 **Supplemental Movie:**
960 **Movie S1:** *A. brasilense* free-swimming wild type cells in the root hair zone of wheat seedlings.
961 The movie is real time and taken from cells inoculated into the slide-in-chamber, 24 hpi.
962

Table 1: Strains and plasmids used in this study.

Strains/ Plasmids	Relevant genotype/ phenotype	Citation
Plasmids		
pHR GFP	Tc_R, constitutive GFP expression	(39)
pRK415	broad-host-range vector; Tc_r	(40)
pRKTlp1	pRK415 containing a 2.7kb DNA fragment encompassing promoter and tlp1 ORF; Tc_R	(23)
pRKTlp1$_{R562A\ R563A}$	pRK415 containing a 2.7kb DNA fragment encompassing promoter and tlp1 ORF with a site directed mutations at residues 562 and 563; Tc_R	(23)
pIND4	Inducible expression plasmid, Km_R	(41)
pRED-DGC	pIND4 expressing a red-light activated diguanylate cyclase (bphS-bphO); Km_R	(22)
pBLUE-PDE	pIND4 expressing a red-light activated diguanylate cyclase and a blue-light activated phosphodiesterase (bphS-bphO-eb1); Km_R	(22)
Bacterial Strains		
E. coli		
S17.1 (pHRGFP)	*E. coli* strain carrying pHRGFP	This study
Azospirillum brasilense		
WT	Wild type strain (Sp7); Amp_R	ATCC29145
Δ*tlp1*	*Δtlp1*::Km derivative of Sp7; Amp_R Km_R	(18)

Δaer	WT with Aer receptor deleted using markerless deletion; Amp^R	(24)
⊗che1⊗che4	*Δ(cheA1−cheR1)Δ(cheA4−cheR4)::* Cm (Cm^R), Gm (Gm^R)	(35)
WT (pHR GFP)	Wild type strain (Sp7) carrying pHR GFP; Amp^R Tc^R	This work
WT (pIND4)	Wild type strain carrying pIND4; Amp^R Km^R	(22)
WT (pRED-DGC)	Wild type strain carrying pRed-DGC; Amp^R Km^R	(22)
WT (pBLUE-PDE)	Wild type strain carrying pBLUE-PDE; Amp^R Km^R	(22)
⊗tlp1(pHR GFP)	Δ*tlp1* carrying pHR GFP; Amp^R Km^R Tc^R	This work
Δ*tlp1* (pRK415)	Δ*tlp1* carrying the broad host range vector pRK415; Amp^R Km^R Tc^R	(23)
Δtlp1(pRK Tlp1)	Δ*tlp1* carrying pRKTlp1; Amp^R Km^R Tc^R	(23)
Δtlp1 (pRK Tlp1$^{R562A\ R563A}$)	Δ*tlp1* carrying pRKTlp1$^{R562A\ R563A}$; Amp^R Km^R Tc^R	(23)
Δ*aer* (pRKAer)	Δ*aer* with Aer expressed from pRK415, Amp^R Tet^R	(24)
Δaer(pRKAerΔPilZ)	Δ*aer* with truncated Aer expressed from pRK415, Amp^R Tet^R	(24)
⊗che1⊗che4 (pHR GFP)	carrying pHR GFP; Amp^R Tc^R	This work