Specific Root Exudate 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 alpha-proteobacterium *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 endpoint 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. Furthermore, the bacterial second messenger c-di-GMP modulates these chemotaxis responses. Together, these 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 mediates competitive colonization of root surfaces. Root exudates are complex chemical mixtures that are spatially and temporally dynamic. Identifying the exact chemical(s) that mediates the 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 exudate gradients and identify key chemical signals that shape root surface colonization in different plants and regions of the roots.

KEYWORDS *Azospirillum*, chemotaxis, rhizosphere, root exudates

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 and plant productivity and

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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., see references 5 and 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 that in marine and sediment environments (7, 8). Chemotaxis is initiated when chemicals in the environment bind to membrane-anchored chemotaxis receptors. A repellent-bound chemotaxis receptor causes the phosphorylation of the associated cytoplasmic histidine kinase CheA from ATP. Phosphorylated CheA (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 roles 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 exceptions to this are recent studies that used a microfluidic device to monitor *Bacillus subtilis* behavior and colonization of *Arabidopsis thaliana* roots over time (5, 18). While this device allows plant-bacterium 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 bacterial 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 bacteria 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 2 of these modulating flagellar motility and controlling chemotaxis, and 51 chemotaxis receptors (23, 24). The roles of motility, chemotaxis, as well as other cellular functions in root surface colonization by *A. brasilense* and related species have been previously studied using mutant strains, endpoint 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 about other motile plant-associated bacteria, the emerging picture of events of root surface colonization by *A. brasilense* is relatively static because the establishment and progression of plant root-microbe associations are typically derived from discrete, fixed time points, 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 the 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

The 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 that allows plants and bacteria to be monitored spatially and temporally,

FIG 1 Interaction of WT and nonchemotactic ($\Delta che1 \Delta che4$) *A. brasilense* with wheat roots in the slide-in chamber. WT and chemotaxis-null ($\Delta che1 \Delta che4$) bacteria carrying pHRGFP to constitutively express GFP were mixed with the semisolid 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 in red. Shown are WT (A) and $\Delta che1 \Delta che4$ (B) accumulations close to the root surface in the root hair and elongation zones. Bands of motile fluorescent bacteria are denoted by brackets, and the average band widths in micrometers are noted below the pictures. Images are representative of data from 3 biological replicates in different chambers.

undisturbed, for up to 1 week using confocal microscopy (see Fig. S1 in the supplemental material) (32). The chamber consists of an 85-mm by 65-mm by 8-mm rectangle with a 62-mm by 20-mm rectangular opening. Three- to five-day-old sterile seedlings can be planted in the 21-mm by 5-mm opening at the top of the chamber. A slide is secured on the back of the device (Fig. S1D), and a coverslip is secured on the front (Fig. S1B), to create an internal cavity with a volume of ~ 3 ml. The internal grid allows the monitoring of specific areas over time. We filled the chamber with a semisolid (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 $\sim 10^7$ cells/ml in 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 to 5 h postinoculation (hpi). 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 green fluorescent protein (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 hpi compared to a zone away from the root (Fig. S2). Cell numbers around the root increased over time (Fig. 1A and Fig. 2). At 24 hpi, the WT accumulated in tight bands within $150 \mu\text{m}$ of the root surface in the root hair and elongation zones (Fig. 1A, brackets). Within these bands, cells swam in long runs, with less frequent changes in direction, but on the edges of or outside the bands, cells frequently reversed and swam in shorter runs (Movie S1). The 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 and

FIG 2 Quantification of *A. brasilense* colonization levels on wheat roots. Colonization levels in each root zone were determined by measuring the fluorescence of surface-attached cells normalized to the area of the root observed and expressed in arbitrary units (a.u.). The calculated total corrected fluorescence (CTCF) for each channel was calculated as follows: integrated density/(area of selection $\times$ mean fluorescence of the background). *t* tests were used to determine if WT colonization differed significantly from $\Delta che1 \Delta che4$ colonization. * indicates a significant difference from the $\Delta che1 \Delta che4$ strain ($P \leq 0.05$).

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 ($\Delta che1 \Delta che4$). Cells of the $\Delta che1 \Delta che4$ strain are fully motile but are unable to perform chemotaxis (23, 24). We found that 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. 2). In fact, the cell distribution near the roots was low regardless of the areas of the roots considered. Therefore, a nonchemotactic 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 nonmotile 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). Nonchemotactic 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 on *Medicago sativa* (alfalfa) roots, another plant that *A. brasilense* is known to colonize (15), in the slide-in chamber. The WT accumulated in the root hair and elongation zones of alfalfa, but it did not accumulate at the root tip (Fig. 3A and C). Chemotaxis-null cells ($\Delta che1 \Delta che4$) 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 postinoculation, which was delayed compared to *A. brasilense* colonization of wheat, which was observed at 48 hpi (compare Fig. 2 to Fig. 3C and D). When placed in competition, the WT was able to colonize alfalfa roots, whereas the $\Delta che1 \Delta che4$ 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

FIG 3 WT and chemotaxis-null ($\Delta che1 \Delta che4$) behaviors in the presence of alfalfa roots. (A and B) Free-swimming WT (A) and $\Delta che1 \Delta che4$ (B) cell accumulation around the alfalfa roots in the slide-in chamber. Shown is the time course of colonization of alfalfa roots by the WT (A) and $\Delta che1 \Delta che4$ (B) strains in the slide-in chamber. *A. brasilense* bacteria constitutively expressing GFP were inoculated into a slide-in chamber containing 3-day-old wheat and imaged over the course of 72 h postinoculation (hpi). *A. brasilense* is visible in green, and the wheat roots are in red (autofluorescence). Images are representative of results from 3 chamber replicates. (C) In-chamber colonization levels were quantified by calculating the CTCF of the attached bacteria and normalizing to the root area. (D) Five-day colonization was measured by CFU counts from homogenized roots. A *t* test indicated no significant difference from $\Delta che1 \Delta che4$ colonization ($P \leq 0.05$) for colonization in any of the root zones. n.r., no bacteria recovered.

FIG 4 Interaction of wild-type and nonchemotactic ($\Delta che1 \Delta che4$) *A. brasilense* bacteria in the root-in-pool assay with intact roots from wheat and alfalfa seedlings. (A and B) Wheat roots from intact seedlings were placed in a suspension of motile WT (A) or chemotaxis-null mutant (B) strain bacteria, and free-swimming behavior in the vicinity of the roots was observed and recorded over time. WT *A. brasilense* accumulates in a band in the root hair and tip zones (indicated by the white arrows), while the chemotaxis-null strain forms a homogeneous pool that does not change within 10 min of observation. (C) Intensity profile analysis of images shown for the WT cell suspension in the root-in-pool assay at 0, 30, and 60 s. (D) WT and chemotaxis-null strains in the presence of the root tip (left) and root hair zone (right) of alfalfa seedlings in the root-in-pool assay.

assay (the root-in-pool assay [see Materials and Methods]) to monitor bacterial chemotaxis responses upon initial root sensing. In this assay, a root from a seedling that 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 min. In contrast to the slide-in chamber, the root-in-pool assay allows the 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 s), this band of motile cells moved closer to the root surface (Fig. 4A and C). Conversely, at the root tip, WT bacteria formed a band $100 \pm 50 \mu\text{m}$ 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 min of 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 and C). This behavioral pattern suggested that cells responded by chemotaxis by moving away from the repellent(s). The $\Delta che1 \Delta che4$ 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 homogeneous pool of cells. These observations indicate that the 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 postinoculation, but a similar accumulation was not detected in the root-in-pool assay, which spans minutes rather than hours. Together, these data suggest that motile *A. brasilense* bacteria are chemotactically attracted to conditions in the root hair and elongation zones of wheat, which would promote the movement of *A. brasilense* closer to these root surfaces over time and root surface colonization. In contrast, motile *A. brasilense* bacteria are chemotactically repelled by conditions in the root tip zone, to isolate them from this region, preventing surface colonization.

One possible explanation for this difference in cell accumulation near wheat and alfalfa roots is the compositions 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 either are not represented in the exudates of alfalfa or are present but that 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* bacteria 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 acid 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 the wheat and alfalfa total exudate abundances revealed that the exudate abundance profiles of alfalfa and wheat are mostly nonoverlapping (Fig. 5A). We found organic acids and amino acids to be broadly represented in the exudates of both wheat and alfalfa roots, but their distributions in each plant root exudate sample were different. Specifically, PCA indicated that while the organic acid abundance profiles of wheat and alfalfa were unique (Fig. 5B and Fig. S4), the amino acid abundance profiles of wheat and alfalfa were overlapping (Fig. 5C). These analyses suggest that the organic acid, 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 behaviors in the wheat and alfalfa rhizospheres. Given that *A. brasilense* is preferentially attracted to organic acids, while amino acids are weak attractants (14, 34–36), 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 the relative abundances of organic acids, alfalfa exudates had higher abundances of several organic acids than did wheat exudates (Fig. S4). The contribution of the organic acid profiles in root exudates to *A. brasilense* chemotaxis in the wheat versus the alfalfa rhizosphere is thus not straightforward. *A. brasilense* utilizes organic acids as preferred carbon sources, while amino acids are poor carbon sources (14,

FIG 5 Principal-component analysis (PCA) of metabolites detected in wheat and alfalfa root exudates. (A) PCA loading plot of the $\log_{10}$ abundance of total metabolites isolated from three biological samples of wheat and alfalfa. The 95% confidence intervals of the PCA scores' covariances from three samples of wheat and alfalfa are represented as ellipses. (B) PCA of only organic acids isolated from three biological samples of wheat and alfalfa. The 95% confidence intervals of the PCA scores' covariances from 3 samples of wheat and alfalfa are represented as ellipses. (C) PCA loading plot of $\log_{10}$ abundances of only amino acids isolated from three biological samples of wheat and alfalfa. The 95% confidence intervals of the PCA scores' covariances from 3 samples of wheat and alfalfa are represented as ellipses.

34–36), making it possible that the 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 than in alfalfa exudates in the presence of bacteria, while amino acids were depleted from both root exudates with somewhat similar patterns. Alternatively, the observed changes in the abundances of these chemicals in the presence of cells may result from changes in the 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 chemotactic responses of *A. brasilense* to the wheat and alfalfa rhizospheres is likely more complex. These differences could result from differences in local gradients of identified compounds, the presence of other, undetected compounds, the metabolism of exuded compounds, or a combination of these factors.

A chemoreceptor, Tlp1, mediates attractant responses to organic acids and wheat root hair and elongation zones. The results described above indicate that *A. brasilense* chemotaxis mediates the accumulation of motile cells around wheat roots, which 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* $\Delta tlp1$ mutant derivative in the slide-in-chamber assay. We found that the *A. brasilense* $\Delta tlp1$ mutant derivative did not accumulate in the root hair and elongation zones (Fig. 2 and 6), 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 the attractant signal(s) in the root hair and elongation zones while also increasing the 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 $\Delta tlp1$ mutant derivative behaved similarly in the short-term assay as in the longer-term slide-in-chamber assay. In this assay, $\Delta tlp1$ 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 $\Delta tlp1$ mutant was also impaired in colonizing the corresponding regions of the root surfaces, as the bacterial density in the root hair and elongation zones showed large variations, which suggested that cells were impaired in the ability to permanently colonize these regions. On the other hand, the cell density increased at the surface of the root tips, in contrast to the wild-type colonization pattern (Fig. 2 and 6).

Previous work has shown that Tlp1 mediates the 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-plug assay (Fig. S5). WT cells exhibited

FIG 6 *A. brasilense* $\Delta tlp1$ mutant strain free-swimming response and colonization of wheat roots. (A) $\Delta tlp1$ strain in the slide-in chamber with wheat seedlings. Shown is the time course of the colonization of wheat root surfaces by the *A. brasilense* $\Delta tlp1$ mutant strain. The chambers were filled with Fahraeus medium containing *A. brasilense* constitutively expressing GFP (pHRGFP) and imaged over the course of 72 h. *A. brasilense* is visible in green, and the wheat roots are in red. Images are representative of data from 3 biological replicates in different chambers. Colonization levels were quantified by calculating the CTCF for each channel as follows: integrated density/(area of selection $\times$ mean fluorescence of the background). A *t* test indicated no significant difference from WT *A. brasilense* colonization ($P \leq 0.05$) for colonization in any of the root zones. (B) Role of Tlp1 and c-di-GMP binding to Tlp1 in the response to wheat roots. A root-in-pool assay was used to observe the behavior of cells lacking Tlp1 ($\Delta tlp1$) or expressing Tlp1 impaired in binding to c-di-GMP (Tlp1^{R562A R563A}) in the presence of wheat. Arrows indicate the accumulation of motile cells at that position.

attractant responses to spatial gradients of malate, pyruvate, and succinate, and this response was absent in a motile but nonchemotactic 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 that 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 the presence of *A. brasilense*, malate, as well as other organic acids, was also rapidly depleted from wheat root 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 zone 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 accumulate 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) (37), 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 min 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 min, 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. $\Delta tlp1$ cells did not respond to hydrogen peroxide gradients, but the repellent response was restored by expressing Tlp1 in *trans* in the $\Delta tlp1$ mutant strain background (Fig. 7A). Despite differences in chemotaxis responses, both the WT and $\Delta tlp1$ strains had similar susceptibilities to killing by hydrogen peroxide, with 3% hydrogen peroxide killing the WT (9.9 ± 1.4 -mm zone of inhibition) and $\Delta tlp1$ (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 described 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 $\Delta tlp1$ mutant, the Δaer mutant failed to chemotactically respond to spatial gradients of hydrogen peroxide in the ROS disc assay, and the repellent response was restored by expressing 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 chemotaxis responses of the $\Delta tlp1$ or Δaer mutant, each expressing variants of Tlp1 and Aer, unable to bind c-di-GMP, i.e., Tlp1^{R562A R563A} (38) or Aer Δ PilZ (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 Aer Δ PilZ-expressing strain had great variability in the distance migrated (0.46 ± 0.15 cm) and the strength of the response (rings were frequently fainter than those for a WT response). The responses of both

FIG 7 Roles of PilZ domain chemoreceptors and c-di-GMP levels in the response to ROS gradients. (A and B) Motile *A. brasilense* $\Delta tlp1$ (A) or Δaer (B) bacteria were exposed to gradients generated by filter paper soaked in buffer, hydrogen peroxide, or cumene peroxide. (C and D) To determine the role of intracellular c-di-GMP levels in the response to ROS gradients, c-di-GMP levels were manipulated using an optogenetic plasmid system, as described in Materials and Methods. WT *A. brasilense* cells with a red-light-activated diguanylate cyclase (pRED-DGC) or blue-light-activated phosphodiesterase (pBLUE-PDE) were illuminated with green (control), red, or blue 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 black arrows pointing at the ring formed by bacteria. The plates were observed every 5 min.

mutants were compromised since the cells consistently produced “blebs” at various distances from the discs (Fig. 7A and B), suggesting that the response to the hydrogen peroxide gradient is only partially restored and/or that the cell population is heterogeneous in the level of expression of the constructs. Furthermore, both of these mutants had weak responses, as rings were not as evident as those of the WT.

PilZ domains bind c-di-GMP (39–41). 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 time scale consistent with chemotaxis (42). This system consists of a plasmid constitutively expressing a red-light-activated diguanylate cyclase and a plasmid expressing a blue-light-activated phosphodiesterase (42, 43). Our previous work showed that illuminating cells carrying these plasmids expressing these proteins with red or blue light for 10 s 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 the c-di-GMP level is transient, lasting less than 10 min (42, 43). Lowering the 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 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 the expression of an Aer Δ PilZ variant in the Δ aer background not only failed to rescue discrete attractant and repellent responses to distinct wheat root regions but also abolished the ability of cells to navigate gradients originating from roots, and it also produced additional detrimental effects that suggested a complete lack of chemotaxis signaling, resulting in these cells behaving similarly to a nonchemotactic 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 the expression of a variant of Tlp1 with a defective PilZ domain (Tlp1^{R562A R563A}) would produce chemotaxis defects similar to those that we previously observed with the Δ aer strain expressing Aer Δ PilZ. To test this hypothesis, we analyzed the behavior of the *A. brasilense* Δ tlp1 mutant strain expressing either parental Tlp1 or a variant unable to bind to c-di-GMP (Tlp1^{R562A R563A}) (38) in the root-in-pool assay (Fig. 6B). While the expression of parental Tlp1 restored the wild-type behavior, the expression of the Tlp1^{R562A R563A} 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 nonchemotactic mutant strain, while the Δ tlp1 strain still responded to the root tips (Fig. 6B). 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 the expression of Tlp1 or Aer variants with nonfunctional PilZ domains have on chemotaxis signaling further support the hypothesis that 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* on different plants. The 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, particularly 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 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, but not alfalfa, exudates were exposed to *A. brasilense*. Given the short time of exposure of bacteria to the exudates, the known metabolic capacity of *A. brasilense* bacteria, their subsequent behavioral responses, and our bulk analysis of root exudate compositions, we surmise that the 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 revealed a 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 that 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 exudate 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 (44). In *Pseudomonas putida*, the abundance of chemoreceptor transcripts changes with growth conditions and in the presence of maize root exudates (45, 46). Previous work in our laboratory demonstrated that the abundance and contribution of at least 1 of the 51 chemoreceptors encoded in the genome of *A. brasilense* to the chemotaxis response changed with combined nitrogen availability to promote the navigation of air gradients compatible with nitrogen fixation (30). It is thus possible that root exudates modulate the abundance of at least some chemoreceptors to promote cell accumulation in the vicinity of roots, although this remains to be experimentally tested.

The results obtained here also identify c-di-GMP as a major regulator of PilZ domain-containing chemoreceptor signaling activity in the vicinity of wheat roots. The $\Delta tlp1$ or Δaer strain expressing Tlp1 or Aer variants unable to bind c-di-GMP lacked attractant and repellent responses in the wheat rhizosphere, although previous work showed that these strains displayed chemotaxis responses to gradients of a single chemoeffector such as oxygen or malate (31, 38, 42, 47). Similarly, functional PilZ domains, able to bind c-di-GMP, were required for the repellent response to hydrogen peroxide mediated by Tlp1 and Aer. These observations suggest that functional PilZ domains and c-di-GMP binding to these domains on chemoreceptors are necessary for recognizing and integrating cues from complex gradients found in the vicinity of plant roots. How could c-di-GMP binding to one chemoreceptor modulate chemotaxis to complex cues such as those found in the wheat rhizosphere while permitting chemotaxis to gradients of a single chemoeffector? One possibility may lie in the organization of chemoreceptors in large ordered arrays that are clustered at the cell poles

(48). *A. brasilense* possesses two spatially distinct membrane-bound chemotaxis arrays at the cell poles with chemoreceptors segregating into arrays based on heptad repeat length (36H and 38H) (49, 50). The genome of *A. brasilense* encodes 6 PilZ domain-containing chemoreceptors, which all belong to the 38H length class and are thus expected to be clustered together into a single array, together with other 38H chemoreceptors and spatially segregated from the 36H chemotaxis signaling array (49). In chemotaxis signaling arrays, chemoreceptors are allosterically coupled (51). With such a spatial arrangement, the deletion of a single PilZ domain-containing chemoreceptor, such as in the $\Delta tlp1$ and Δaer 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 adopt a conformation that abolishes signaling competence by the chemotaxis array. Given that the majority of *A. brasilense* chemoreceptors belong to the 38H class (49), 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 the lack of c-di-GMP binding to Tlp1 (38, 42) 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 conformational changes when binding to c-di-GMP (39, 52–54). Given the allosteric packing and interaction of chemoreceptors in chemotaxis signaling arrays, it is conceivable that the binding of c-di-GMP to PilZ domain chemoreceptors could influence the 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 (49).

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 (38, 42). Changes in c-di-GMP levels with metabolism may thus contribute to *A. brasilense* metabolism-dependent chemotaxis responses. The only previously reported repellents for *A. brasilense* were redox-active compounds such as quinones or inhibitors of the electron transport chain that were thought to directly interfere with energy-sensing chemoreceptors (34). Here, we identify hydrogen peroxide as a repellent for *A. brasilense*. While redox-active compounds may directly alter the signaling activity of chemoreceptors that possess redox motifs such as flavin adenine dinucleotide (FAD) in their sensing domains, as present in Aer or AerC (30, 31), our results here suggest that the repellent effect of hydrogen peroxide on *A. brasilense* chemotaxis may be mediated through changes in c-di-GMP levels that would alter the conformation and, thus, the signaling activity of PilZ-containing chemoreceptors. These results thus also support a role for c-di-GMP in coupling metabolism with chemotaxis in *A. brasilense*.

ROS are critical for plant growth, and root tips are actively growing regions that are known for producing ROS (55, 56). Our results show that *A. brasilense* is chemotactically repelled by hydrogen peroxide, which could be produced by wheat root tips. Our results do not establish that *A. brasilense* senses root-generated hydrogen peroxide, but they support a model in which motile *A. brasilense* bacteria use chemotaxis to avoid accumulating in root zones likely to generate toxic ROS. Previous work has shown that inoculation of *A. brasilense* into wheat root seedlings modulates the production of ROS such as superoxide and the activity of plant enzymes that mitigate ROS stress (37). Furthermore, successful colonization of plant roots by *A. brasilense* depends on the ability of the bacteria to overcome oxidative stress (57). Together, these data suggest that the ability to sense and respond to ROS is critical for the association of *A. brasilense*

with plant roots. *Helicobacter pylori* is also able to sense host-generated ROS, including hydrogen peroxide, by chemotaxis, and this response is required for the ability of the bacterium to colonize the gastric epithelial glands (58). In *H. pylori*, the sensing of oxidative stress and ROS is mediated by the C-terminal CZB domain of the cytoplasmic TlpD receptor (59). The C-terminal CZB domain binds zinc (60), with homologs found at the C termini of chemoreceptors of bacterial pathogens that colonize mucosal surfaces, suggesting that its role in sensing ROS may be widespread (58). Interestingly, TlpD also forms an autonomous chemotaxis signaling cluster that mediates chemotaxis responses to oxidative stress (59). 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 (60), functions to couple chemotaxis signaling to metabolism via the 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, members of 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 genomes of soil bacteria (61). Chemotaxis to organic acids has also been implicated in the association of diverse bacteria with the roots of plants (13, 14, 62, 63). 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 the roots of plants, they experience gradients due to root exudates, which not only change their metabolism through the 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 root surfaces.

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 medium for *A. brasilense* (MMAB) containing 10 mM malate as a carbon source (64) or 1.5% agar tryptone-yeast (TY) medium with the following appropriate antibiotics: ampicillin (200 $\mu\text{g}/\text{ml}$), kanamycin (30 $\mu\text{g}/\text{ml}$), and/or tetracycline (10 $\mu\text{g}/\text{ml}$). For liquid cultures, all *A. brasilense* strains were grown with shaking (180 rpm) in MMAB with 10 mM malate as a carbon source, 20 mM ammonium chloride as a nitrogen source, and appropriate antibiotics.

Generation of GFP-expressing *A. brasilense*. To generate constitutively fluorescent strains, we used the broad-host-range vector pHRGFP (Table 1). pHRGFP was transferred into the Sp7 (WT), Δtlp1 , and $\Delta\text{che1 } \Delta\text{che4}$ strains using biparental conjugation as previously described (64). Transconjugants were selected for on MMAB containing tetracycline and confirmed using a Nikon Eclipse 80i fluorescence microscope equipped with a Nikon CoolSnap HQ2 cooled charge-coupled-device (CCD) camera.

Germination of *T. aestivum* and *M. sativa*. *T. aestivum* (wheat) and *M. sativa* (alfalfa) were utilized throughout this study. *T. aestivum* was 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 h to germinate. Next, plates were placed in the light and allowed to grow for 24 h. All assays were performed on germinated seedlings that were 3 to 5 days old.

Slide-in-chamber dimensions and assembly. The microscope slide-in chamber (85 mm by 35 mm by 8 mm) (catalog no. EH4001; Kerafast) was designed in Autodesk Inventor and printed from an ABS thermoplastic filament with a Fortus 250MC three-dimensional (3D) printer. The print layer height was set to 0.007 in. (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 chemotaxis (Che) buffer (1.7 g liter⁻¹ dipotassium phosphate, 1.36 g liter⁻¹ monopotassium phosphate, 0.1 mM EDTA) suspended in Che buffer, and mixed by repeated inversion in a 1:1 ratio with molten Fahraeus semisolid medium (33) (CaCl₂ at 100 mg liter⁻¹, MgSO₄·7H₂O at 120 mg liter⁻¹, KH₂PO₄ at 100 mg liter⁻¹, Na₂HPO₄ at 150 mg liter⁻¹, and ferric citrate at 5 mg liter⁻¹, with 0.3% agar). Molten Fahraeus medium was used to plug the bottom of the chamber, and the bacterium-Fahraeus medium mixture was then 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 postinoculation. Between 5 and 10 chambers were observed under each inoculation condition.

TABLE 1 Strains and plasmids used in this study

Plasmid or bacterial strain	Description or relevant genotype/phenotype ^a	Reference or source
Plasmids		
pHRGFP	Constitutive GFP expression; Tc ^r	67
pRK415	Broad-host-range vector; Tc ^r	68
pRKtlp1	pRK415 containing a 2.7-kb DNA fragment encompassing the promoter and <i>tlp1</i> ORF; Tc ^r	38
pRKtlp1 ^{R562A R563A}	pRK415 containing a 2.7-kb DNA fragment encompassing the promoter and <i>tlp1</i> ORF with site-directed mutations at residues 562 and 563; Tc ^r	38
pIND4	Inducible expression plasmid; Km ^r	69
pRED-DGC	pIND4 expressing a red-light-activated diguanylate cyclase (<i>bphS-bphO</i>); Km ^r	43
pBLUE-PDE	pIND4 expressing a red-light-activated diguanylate cyclase and a blue-light-activated phosphodiesterase (<i>bphS-bphO-eb1</i>); Km ^r	43
Bacterial strains		
<i>Escherichia coli</i> S17.1(pHRGFP)	<i>E. coli</i> strain carrying pHRGFP	This study
<i>Azospirillum brasilense</i>		
Sp7	Wild-type strain; Amp ^r	ATCC 29145
Sp7 Δ tlp1	Δ tlp1::Km derivative of Sp7; Amp ^r Km ^r	29
Sp7 Δ aer	WT with Aer receptor deleted using markerless deletion; Amp ^r	31
Sp7 Δ che1 Δ che4	Δ (cheA1–cheR1) Δ (cheA4–cheR4)::Cm-Gm; Cm ^r Gm ^r	49
Sp7(pHRGFP)	Wild-type strain carrying pHRGFP; Amp ^r Tc ^r	This work
Sp7(pIND4)	Wild-type strain carrying pIND4; Amp ^r Km ^r	42
Sp7(pRED-DGC)	Wild-type strain carrying pRED-DGC; Amp ^r Km ^r	42
Sp7(pBLUE-PDE)	Wild-type strain carrying pBLUE-PDE; Amp ^r Km ^r	42
Sp7 Δ tlp1(pHRGFP)	Δ tlp1 mutant carrying pHRGFP; Amp ^r Km ^r Tc ^r	This work
Sp7 Δ tlp1(pRK415)	Δ tlp1 mutant carrying the broad-host-range vector pRK415; Amp ^r Km ^r Tc ^r	38
Sp7 Δ tlp1(pRKtlp1)	Δ tlp1 mutant carrying pRKtlp1; Amp ^r Km ^r Tc ^r	38
Sp7 Δ tlp1(pRKtlp1 ^{R562A R563A})	Δ tlp1 mutant carrying pRKtlp1 ^{R562A R563A} ; Amp ^r Km ^r Tc ^r	38
Sp7 Δ aer(pRKAer)	Δ aer mutant with Aer expressed from pRK415; Amp ^r Tet ^r	31
Sp7 Δ aer(pRKAer Δ PilZ)	Δ aer mutant with truncated Aer expressed from pRK415; Amp ^r Tet ^r	31
Sp7 Δ che1 Δ che4(pHRGFP)	Δ che1 Δ che4 mutant carrying pHRGFP; Amp ^r Tc ^r	This work

^aORF, open reading frame.

Root-in-pool assay. Five milliliters of cells was grown to an optical density at 600 nm (OD₆₀₀) of 0.4 in MMAB containing malate as a carbon source. After growing, cells were washed 3 times with Che buffer (1.7 g liter⁻¹ dipotassium phosphate, 1.36 g liter⁻¹ monopotassium phosphate, 0.1 mM EDTA) and resuspended 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, 400 μ l of washed bacteria was used to fill the chamber created by the dip and washer, and a coverslip was then used to cover the pool chamber (31).

All root-in-pool assays were recorded using the 4 $\times$ objective of a Nikon E200 phase-contrast microscope equipped with a Nikon Coolpix digital camera. Assays were observed for 15 min, and the first 3 min were filmed using the Nikon Coolpix digital camera. Fiji was used for all image and video analyses (65). All root-in-pool assay videos were converted to an 8-bit format, 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 s and plotted. The root-in-pool assays were repeated between 3 and 5 times for each strain analyzed.

Fluorescence microscopy and image analysis. All chambers were imaged using a Zeiss LSM710 confocal microscope. Images were analyzed using Fiji (65). 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. The calculated total corrected fluorescence (CTCF) for each channel was calculated as follows: integrated density/(area of selection $\times$ mean fluorescence of the background). Bacterial density and colonization were determined by dividing the CTCF of the GFP signal by the 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 the WT, Δ tlp1, and Δ che1 Δ che4 strains. A *P* value of <0.05 was used to determine significance.

Long-term colonization. Long-term colonization (5 days) was determined using a previously described assay (23). Cells were grown to an OD₆₀₀ of 0.5, and 2 ml of the culture was washed with Che buffer (1.7 g liter⁻¹ dipotassium phosphate, 1.36 g liter⁻¹ monopotassium phosphate, 0.1 mM EDTA) and concentrated in 400 μ l of Che buffer. A 6.5-cm-diameter growth container was filled with 50 ml semisolid Fahraeus medium and allowed to solidify. Four plants (germinated, as described above) were placed in the medium at the edges of the chamber. Twenty microliters of the washed and concentrated bacteria was 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, and homogenized in 400 μ l 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 were grown at 28°C for 2 days and then counted. Colonization efficiency was quantified as follows: $\log(\text{CFU}_{\text{recovered}})/\log(\text{CFU}_{\text{input}})$.

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 h in the plates in the dark, and the water was collected, filter sterilized using a 0.45- μm 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 h. After 24 h, wild-type *A. brasilense* was inoculated into each well of the plate and allowed to incubate for 30 min at room temperature. Before inoculation, wild-type (Sp7) *A. brasilense* was grown to exponential phase ($\text{OD}_{600} = 0.4$ to 0.8); cells were collected, washed with sterile water, and concentrated to an OD_{600} of 1; and 500 μl of concentrated cells was used for the inoculum for each well. After collection, exudates were filter sterilized using 0.45- μm filters, 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-bsmmmc/>). The lyophilized exudates were resuspended in 1 ml of water and separated by using a Hydro reverse-phase (RP) high-performance liquid chromatography (HPLC) instrument. Samples were ionized by electrospray in negative mode on an Orbitrap mass spectrometer. Data were processed and peaks were picked via Maven software. Metabolite area counts were normalized to the dry sample weight. The heat map for relative abundance was generated by taking the $\log_{10}$ of the normalized area for each of 3 biological replicates and averaging. The fold change was calculated by dividing the average abundance of each compound under various conditions and then $\log_2$ transforming. Principal-component analysis (PCA) on the $\log_{10}$ of exudate abundances of wheat and alfalfa triplicates was performed using a customized Python algorithm with the scikit-learn package (66).

Chemical-in-plug assay. To generate chemicals in plugs, malate (10 mM), pyruvate (10 mM), or succinate (10 mM) in sterile water was 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 4 $\times$ objective of a Nikon E200 phase-contrast microscope. Behavior was videoed using a C-mounted Nikon Coolpix digital camera for 5 min. Slices were obtained from videos using Fiji.

Spatial gradient assay for ROS chemotaxis. Twenty-five milliliters of cells was grown to an OD_{600} of 0.5 in TY liquid medium with appropriate antibiotics. The entire culture was washed with Che buffer, resuspended in 25 ml of Che buffer, and mixed with 25 ml of TY semisolid (0.3% agar) medium (1:1, vol/vol). Twenty-five milliliters of the bacterium-semisolid TY mixture was poured into square petri dishes. Sterilized filter paper discs soaked in 20 μl hydrogen peroxide, cumene peroxide, or Che buffer were placed on top of the bacterium-agar mixture, and the chemotactic response was monitored every 5 min for 2 h. 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) (42, 43), cultures were grown to an OD_{600} of 0.5 in TY liquid medium with appropriate antibiotics (Table 1) and maintained in the dark, and plates were exposed to red light (610 to 730 nm) or blue light (450 nm) for 30 s using a lamp equipped with a Magic Lighting lightbulb and remote control immediately before exposure to filter paper. After exposure, plates were maintained in the dark, and noninducing green light (505 to 575 nm) was used for observing responses and obtaining images. The images were obtained using a Nikon Coolpix digital camera.

Measuring *A. brasilense* sensitivity to hydrogen peroxide via zone-of-inhibition assays. The WT and Δtlp1 strains were grown in TY liquid culture until the OD_{600} reached 0.8. Five hundred microliters of cells was spread onto TY solid medium with ampicillin. Once the plates dried, sterile filter paper discs were soaked in Che buffer or hydrogen peroxide (3%, 0.3%, or 0.03%, vol/vol) (Fisher Scientific) and placed on the plates. After 48 h of incubation at 28°C, zones of growth inhibition were measured.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 0.7 MB.

SUPPLEMENTAL FILE 2, MPG file, 3.8 MB.

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