Azospirillum brasilense Chemotaxis Depends on Two Signaling Pathways Regulating Distinct Motility Parameters

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ABSTRACT

The genomes of most motile bacteria encode two or more chemotaxis (Che) systems, but their functions have been characterized in only a few model systems. Azospirillum brasilense is a motile soil alphaproteobacterium able to colonize the rhizosphere of cereals. In response to an attractant, motile A. brasilense cells transiently increase swimming speed and suppress reversals. The Che1 chemotaxis pathway was previously shown to regulate changes in the swimming speed, but it has a minor role in chemotaxis and root surface colonization. Here, we show that a second chemotaxis system, named Che4, regulates the probability of swimming reversals and is the major signaling pathway for chemotaxis and wheat root surface colonization. Experimental evidence indicates that Che1 and Che4 are functionally linked to coordinate changes in the swimming motility pattern in response to attractants. The effect of Che1 on swimming speed is shown to enhance the aerotactic response of A. brasilense in gradients, likely providing the cells with a competitive advantage in the rhizosphere. Together, the results illustrate a novel mechanism by which motile bacteria utilize two chemotaxis pathways regulating distinct motility parameters to alter movement in gradients and enhance the chemotactic advantage.

IMPORTANCE

Chemotaxis provides motile bacteria with a competitive advantage in the colonization of diverse niches and is a function enriched in rhizosphere bacterial communities, with most species possessing at least two chemotaxis systems. Here, we identify the mechanism by which cells may derive a significant chemotactic advantage using two chemotaxis pathways that ultimately regulate distinct motility parameters.

Bacterial chemotaxis provides a competitive advantage by guiding motile cells in gradients of chemoeffectors toward environments that support growth and metabolism. Chemotaxis contributes to the establishment of various associations of bacteria with eukaryotic hosts (mammals, insects, and plants) and promotes virulence, symbiosis, and the establishment of microbial communities (1). Bacterial chemotaxis and motility are widespread traits encoded in the genomes of bacteria inhabiting diverse environments, and these functions are specifically enriched in microorganisms found in soils (2), suggesting that they provide a significant competitive advantage in this environment. Consistent with these findings, comparative genome analyses of chemotaxis in diverse motile bacteria suggested that most bacteria possess two chemotaxis systems and soil-dwelling bacteria often have more than two chemotaxis systems (3).

The molecular mechanism of chemotaxis signal transduction has been deciphered in most detail in the model organism Escherichia coli, which possesses a single chemotaxis system. In E. coli, the chemotaxis signal transduction pathway consists of membrane-bound receptors clustered in dense arrays at the cell poles, where their C-terminal domains associate with cytoplasmic CheA kinase and the CheW scaffolding protein. When stimulated by a repellent, CheA autophosphorylates on a conserved histidine residue (H48) using ATP and transfers its phosphate to the aspartate residue (D57) of CheY (1). Phospho-CheY binding to flagellum motors with a high affinity triggers a switch in the direction of flagellum motor rotation from counterclockwise to clockwise. This event causes a change in the swimming direction of the cell or a tumble (1). A phosphatase, CheZ, assists signal termination by acting on phospho-CheY (1). In addition, a receptor-specific methyltransferase, CheR and a receptor-specific methylesterase, CheB, activated by phosphotransfer from phospho-CheA, differentially methylate the receptors to reset sensitivity (1). This basic set of chemotaxis proteins comprises a signaling pathway that is generally conserved across bacterial species (3). However, there are notable exceptions to this paradigm, such as the absence of CheB and CheR in some species, the existence of multiple CheY response regulators in others, or the presence of ancillary chemotaxis proteins not found in E. coli (1). Another variation on this theme is the presence of multiple chemotaxis pathways in the genomes of most motile bacteria (3). Some of these additional chemotaxis pathways regulate type IV pilus-dependent motility or alternative cellular functions (ACFs), and they can be identified...
due to their unique structure (3). Most chemotaxis pathways found in bacterial genomes are predicted to regulate flagellar motility patterns. The contribution of multiple chemotaxis systems to the regulation of changes in the swimming direction was demonstrated in some species. For example, in Rhodobacter sphaeroides, two chemotaxis systems together control the probability of stops in flagellar rotation (4).

Azospirillum brasilense is a motile soil bacterium that inhabits the rhizosphere of diverse plant species. A. brasilense cells swim using a single polar flagellum and change swimming direction when the direction of flagellar rotation briefly reverses, causing cells’ movement to be redirected in a different direction (5). In addition to regulating the probability of swimming reversals, motile A. brasilense cells navigating an attractant gradient can transiently increase their swimming speed (6). The available genome sequence of A. brasilense indicates the presence of four distinct chemotaxis operons, three (che1, che2, and che3) of which are also present in the genomes of all Azospirillum strains sequenced to date as well as in the genome of the closely related organism Rhodospirillum centenum (7–10), suggesting that they were present in the last common ancestor of these two genera (Fig. 1). The chemotaxis pathway that regulates transient increases in swimming speed in response to attractants has been identified to be Che1 (6).

The existence of a distinct pathway for controlling swimming reversals comes from the observation that inactivation of cheB1 or cheR1 impaired the ability to regulate the probability of swimming reversals but mutations in either che1, cheA1, or cheY1 did not (11). These data were interpreted to suggest that Che1 functionally interacts with the unidentified pathway controlling the probability of swimming reversals (6). However, the chemotaxis pathway(s) responsible for controlling changes in the direction of flagellar rotation to trigger reversals is not yet known. The role of che2 in A. brasilense is unknown, but it is a homolog to the che2 operon controlling flagellar biosynthesis in R. centenum (10). In A. brasilense, che2 does not appear to be expressed under standard laboratory conditions (Z. Xie and G. Alexandre, unpublished data). Che3 is a chemotaxis-like ACF pathway that was recently implicated in the control of flocculation in A. brasilense (12).

che4 is present in the genomes of all Azospirillum strains sequenced to date but absent from the R. centenum genome. In R. centenum, the Che1 chemotaxis system controls all chemotaxis responses (13); in contrast, its homolog in A. brasilense (Che1) controls swimming speed and has only a minor role in chemotaxis. The Che4 system is thus the most likely candidate for controlling the probability of swimming reversals in this species. Here, we show that Che4 is essential for all chemotaxis responses in A. brasilense and for competitive wheat root surface colonization. We demonstrate that the signaling output from Che4 directly modulates the probability of reversals and that signaling from Che1 and Che4 is integrated during chemotaxis to produce an enhanced response to attractants. These results illustrate a novel mechanism by which motile bacteria utilize two chemotaxis pathways regulating distinct motility parameters to alter movement in gradients and to increase their chemotactic advantage.

FIG 1 Chemotaxis gene clusters within the A. brasilense genome. Boxes represent open reading frames and are drawn to scale. The chemotaxis genes within each cluster were either previously characterized or identified by homology searches, as detailed in the text. orf, open reading frame; RR, response regulator gene; HK, histidine kinase gene; mcp, gene for methyl-accepting chemotaxis protein.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The bacterial strains used in this study are listed in Table 1. The A. brasilense strains were grown at 28°C with shaking (200 rpm). Minimal medium for A. brasilense (MMAB) was prepared as described previously (14, 15). Cells were induced for nitrogen fixation by first growing them in MMAB, followed by three to four washes of the cell pellet in sterile chemotaxis buffer (10 mM phosphate buffer [pH 7.0], 1 mM EDTA) (11) by centrifugation. The pellet was then resuspended in MMAB lacking any nitrogen source. Nitrogen fixation was induced by growth under these conditions overnight at 28°C without shaking to ensure low aeration conditions. All culture stocks were routinely maintained on tryptone-yeast (TY) medium (per liter, 10 g Bacto tryptone, 5 g yeast extract) or MMAB solidified with 1.5% (wt/vol) agar.

For A. brasilense, the following antibiotics were added at the indicated final concentrations: ampicillin at 200 μg/ml, chloramphenicol at 20 μg/ml, kanamycin at 30 μg/ml, gentamicin at 20 μg/ml, and tetracycline at 10 μg/ml.

Mutagenesis. To construct a strain from which the cheA4 gene was deleted, an 843-bp upstream fragment including 126 bp of cheA4 and a downstream 825-bp sequence including 232 bp at the 3′ end of cheA4 were PCR amplified using primers cheA4Up-F, cheA4Up-R, cheA4Dwn-F, and cheA4Dwn-R (see Table S1 in the supplemental material). These primers were engineered to include 5′ XbaI (cheA4Up-F) and 3′ HindIII (cheA4Dwn-R) restriction sites for cloning into pUC19 as well as BamH1 sites (present at the 5′ end of primer cheA4Up-F and at the 3′ end of primer cheA4Dwn-F) to permit subsequent insertion of a gentamicin resistance (Gm)′ cassette isolated from p34S-Gm by BamH1 restriction digestion (Table 1). After verification of the sequence by sequencing, the ΔcheA4::Gm region present on the pUC19 vector was isolated by restriction digestion with XbaI and HindIII and inserted into the suicide vector pSUP202 (Table 1) that had been digested with the same enzymes, yielding pSUPΔcheA4::Gm. The pSUPΔcheA4::Gm vector was transformed into E. coli S17-1 for biparental mating with strains A. brasilense Sp7 and its ΔcheA1 mutant derivative for allelic exchange, as previously described (15). For constructing a mutant lacking cheY4, an upstream fragment encompassing the first 6 bp of cheY4 and an additional 577 bp of upstream DNA sequence and a downstream fragment including the last base pair of cheY4 and an additional 584 bp downstream of the stop codon were amplified using the primer pair cheY4Up-F and cheY4Up-R and the primer pair cheY4Dwn-F and cheY4Dwn-R (see Table S1 in the supplemental material), which were designed to include restriction sites to facilitate cloning into the EcoRI- and HindIII-digested pUC19 vector. The fragments were also engineered to include a BamH1 site for insertion of a chloramphenicol cassette isolated from a p34S-Cm BamHI-digested vector to yield pKGΔcheY4::Cm (Table 1). The latter was transformed into E. coli S17-1 cells and transferred into A. brasilense Sp7 or its ΔcheY4 mutant derivative by biparental mating for allelic exchange, as described above. PCR was used to verify that the strains carried the appropriate deletions.
TABLE 1 Strains and plasmids used in this study

<table>
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<tr>
<th>Strain or plasmid</th>
<th>Genotype or description</th>
<th>Reference or source</th>
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<tr>
<td><strong>Strains</strong></td>
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<td><em>Azospirillum brasilense</em></td>
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<tr>
<td>AB101</td>
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<tr>
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<td>AB143</td>
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<td>Invitrogen</td>
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<tr>
<td>S17-1</td>
<td>thi endA recA hsdR strain with RP4-2Tc::Mu::Km::Tn7 integrated in chromosome</td>
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To construct a strain from which most of the che4 sequence was deleted, we first used PCR amplification of a 698-bp fragment encompassing the first gene of the che4 cluster (Fig. 1) and an upstream DNA sequence using primers che4Up-F and che4Up-R (see Table S1 in the supplemental material) and a 722-bp fragment overlapping the last gene of che4 (Fig. 1) and including an additional downstream DNA sequence using primers che4Dwn-Up and che4Dwn-R (see Table S1 in the supplemental material). The primers che4Up-F and che4Dwn-F were designed to include 12 bp of overlapping DNA sequence that was used in a second round of splicing by overhang extension PCR (16) to produce a 1,420-bp fusion product between the upstream and downstream PCR fragments. The 1,420-bp product was restriction digested with PstI and XbaI, which were engineered in the PstI and XbaI restriction sites of the plasmid expression vector pRK415 (Table 1) and yield a Δche4::Gm construct cloned in pUC19. After verification by sequencing, the Δche4::Gm region was isolated as a PstI and XbaI fragment which was then cloned into the suicide vector pSUPPOL2SCA (17). The suicide vector carrying the che4 deletion insertion construct was transformed into E. coli strain S17-1, followed by allelic exchange after transfer to *A. brasilense* wild-type strain Sp7 and its Δche1 mutant derivative by biparental mating, as described above. Mutants carrying the correct deletion insertions were identified by PCR.

**Complementation and site-specific mutations of che4 and cheY4 genes.** Functional complementation of the mutant phenotypes was conducted by expressing the parental genes or mutated alleles in trans from the broad-host-range pRK415 vector (Table 1). The genes were cloned in-frame and downstream of the plasmid-borne lac promoter with engineered restriction sites and a ribosome binding site to ensure expression (Table 1) (6). Each gene was amplified from the genomic DNA of the wild-type strain, using a set of forward and reverse primers that are listed in Table S1 in the supplemental material. The genes coding for kinase-inactive CheA4 with the H54Q substitution (CheA4H54Q) and the inactive CheY4 with the D57N substitution (CheY4D57N) were synthesized by GenScript (Piscataway, NJ) and cloned into pUC57 between EcoRI and HindIII to isolate the sequence-verified genes. The isolated DNA fragments were cloned into the pRK415 vector. Recombinant plasmids were isolated from transformed *E. coli* TOP10 cells, prior to being transferred into *E. coli* S17-1 for biparental mating with *A. brasilense* recipient strains, as described above. An empty pRK415 vector was transferred to *A. brasilense* Sp7 and its mutant derivatives for use as controls.

**Behavioral assays.** For the capillary assay for aerotaxis, cells were grown to an optical density of 0.600 nm (OD600) of 0.4 to 0.6 (exponential phase of growth) in MMAB supplemented with malate (10 mM) and ammonium (18.7 mM). Cultures were adjusted to an equivalent number of cells (estimated via OD600 measurements) by dilution in chemotaxis buffer. The cells were gently washed three times with chemotaxis buffer by low-speed centrifugation and resuspended in 100 μl MMAB containing malate (10 mM). All cells remained motile under these conditions. Cells were transferred to an optically flat capillary tube (Vitro Dynamics, Inc., Rockaway, NJ) by immersing a capillary tube into the cell suspension. Aerotaxis was visualized under a light microscope as the formation of a...
stable band of motile cells at a distance from the air-liquid interface (the meniscus). An aerotaxis band typically forms within 2 to 3 min and is stable for a minimum of 25 min under these conditions (18). The assay was performed in triplicate using independent cultures. Images were captured using the 4× objective of a phase-contrast Nikon E200 microscope via a C-mounted Nikon Coolpix digital camera.

For chemotaxis in a spatial gradient, soft agar plates were prepared using MMAB solidified with 0.3% agar and supplemented with malate (10 mM) and ammonium chloride (18.7 mM). Cells were grown as indicated above for aerotaxis, and the cell suspensions were adjusted to an equivalent number of cells, estimated by OD<sub>600</sub> measurements, prior to being inoculated in the center of the soft agar plates. The inoculated plates were incubated at 28°C for 48 h before being photographed. The experiment was performed in triplicate for each strain.

**Computerized motion tracking of free-swimming cells.** Digital movies of free-swimming cells were captured using the 40× objective of a phase-contrast Nikon Eclipse E200 microscope fitted with a Sony Hyper-HAD monochrome camera at a rate of 30 frames per second. The videos were converted to a digital format using IC Capture2 software (The Imaging Source, Charlotte, NC) before being analyzed using CellTrak (version 1.5) software (Motion Analysis, Santa Rosa, CA). Changes in swimming parameters (velocity and reversal frequency) were determined for a minimum of 75 cells recorded from at least two different fields of view and three independent cultures. Velocity is expressed as micrometers per second, and reversal frequency is expressed as the number of swimming reversals per second and per cell.

**Plant inoculation.** Wheat (Triticum aestivum) seeds were surface sterilized and germinated for 3 days in the dark followed by an additional day in the light, as previously described (19). Fifty milliliters of Fahraeus medium (CaCl<sub>2</sub>, 100 mg liter<sup>-1</sup>; MgSO<sub>4</sub>·7H<sub>2</sub>O, 120 mg liter<sup>-1</sup>; KH<sub>2</sub>PO<sub>4</sub>, 100 mg liter<sup>-1</sup>; Na<sub>2</sub>HPO<sub>4</sub>·13H<sub>2</sub>O, 150 mg liter<sup>-1</sup>; ferric citrate, 5 mg liter<sup>-1</sup>;) solidified with 0.6% agar was placed in a 6.5-cm-diameter growth chamber. Four plantlets were placed at an equal distance from each other and from the center of the growth chamber. Strains to be tested were inoculated at the center of the chamber. Eight chambers (with four plants each) were prepared to test each strain. The strains were grown in MMAB supplemented with malate (10 mM) and ammonium chloride (18.7 mM) to an OD<sub>600</sub> of 0.5. Two milliliters of the culture was pelleted, washed 3 times with sterile 0.8% KCl by centrifugation, and resuspended in 40 µl of sterile 0.8% KCl. Twenty microliters of this suspension was inoculated into the center of the growth chamber. The number of cells in the inoculum was determined by counting the numbers of CFU. For competitive root colonization assays, the strains to be compared were prepared as indicated above and mixed in a 1:1 ratio prior to inoculation into the chambers. The chambers were incubated at 25°C for 24 h, after which the plants were removed and the roots were excised. The roots of the four plantlets from a single growth chamber were combined and homogenized in 5 ml 0.8% KCl, followed by serial dilutions and plating on selective medium to determine the numbers of CFU. Root colonization was calculated as a colonization index for the plants inoculated with a single strain and as a competitive index for the plants inoculated with two strains. The colonization index for an individual strain was the log<sub>10</sub>(strain output/input strain), where the number of CFU extracted from roots after incubation was normalized to the number of CFU measured in the inoculated input. The competitive index was a ratio calculated as follows: log<sub>10</sub>(mutant output/wild-type output)/(mutant input/wild-type input).

**Identification of chemotaxis proteins in the complete genome sequence of A. brasilense.** The che1 operon organization was previously described (14). To identify other chemotaxis proteins, we used the sequences of the chemotaxis homologs reported in the genome description of A. brasilense Sp245 (10) as queries to search the genome of A. brasilense Sp7 (BioProject accession number PRNA293508) using the BLAST server (20).

**RESULTS**

**che1 mutants lack chemotaxis and aerotaxis.** Genomic sequence analysis predicted that Che1 is the major chemotaxis system in A. brasilense. To test this hypothesis, we constructed strains lacking cheA4 (ΔcheA4; strain AB401), cheY4 (ΔcheY4; strain AB402), or the entire che4 cluster (Δche4; strain AB403) (Table 1) and tested their chemotaxis and aerotaxis abilities using spatial gradient assays (Fig. 2). All mutants were motile and grew at similar rates in various media and under various incubation conditions (data not shown). While the ΔcheA4 mutant was null for chemotaxis and aerotaxis, the ΔcheY4 and Δche4 mutants had partial defects in chemotaxis and aerotaxis, which were restored by expression of wild-type cheA4 but not cheA4<sup>H54Q</sup> (S145E) (C). Similarly, the aerotaxis defect of the ΔcheY4 mutant strain is rescued by expression of wild-type CheY4 but not CheY4<sup>D57N</sup> (D). In panels B, C, and D, the number of cells was equivalent in all tubes, and all strains were motile. * Formation of a stable band of motile cells.

**Statistical analysis**. For comparing the wild-type and mutant phenotypes (swimming speed, swimming reversals, and plant colonization), we determined average values from at least three independent experiments performed in duplicate and performed one-way analysis of variance (alpha level, 0.05), followed by pairwise two-sample t tests assuming equal variances (alpha level, 0.05) using Prism (version 6) software (GraphPad Software Inc., San Diego, CA).

**FIG 2** Taxis behaviors of A. brasilense and its che1 mutant derivatives. (A) Chemotaxis in the soft agar plate assay containing malate (10 mM) and ammonium chloride (18.7 mM) as the carbon and nitrogen sources, respectively. The strains tested are indicated at the top of each plate. The pictures were taken after 48 h of incubation at 28°C. Representative images from at least five different assays are shown. (B) Aerotaxis in the spatial gradient assay. The air gradient is established by diffusion, in the direction indicated by the arrow, into capillary tubes filled with a suspension of motile cells. The images were taken 5 min after placement of the cells in the capillary tubes. (C and D) Functional complementation of the aerotaxis defects of the Δche4 mutant (C) and of the ΔcheY4 mutant (D) in the capillary assay for aerotaxis. The photographs were taken 10 min after placement of the suspension of motile cells in the capillary tubes. The plasmids carried by the strains are derivatives of the broad-host-range plasmid pRK415. The aerotaxis defect of the Δche4 mutant strain is rescued by expression of wild-type CheA4 but not CheA4<sup>H54Q</sup> (C). Similarly, the aerotaxis defect of the ΔcheY4 mutant strain is rescued by expression of a wild-type CheY4 but not CheY4<sup>D57N</sup> (D). In panels B, C, and D, the number of cells was equivalent in all tubes, and all strains were motile. * formation of a stable band of motile cells.
The ΔcheY4 and Δche4 mutants were null for aerotaxis but displayed residual chemotaxis in the soft agar assay (Fig. 2A and B). Similar patterns were observed when other carbon sources were used in the soft agar assay (data not shown). The different phenotypes of some of these mutants for chemotaxis versus aerotaxis were intriguing. The discrepancy between the behavior of the ΔcheY4 and Δche4 mutants in the aerotaxis assay could be due to the different incubation times and conditions under which these assays were conducted. The aerotaxis assay was performed using a suspension of free-swimming cells placed into a capillary tube. The aerotactic band typically forms within 2 to 3 min. The chemotaxis rings formed in soft agar plates were observed after at least 24 to 48 h of growth. One possibility is that the rings observed in the soft agar plates were the result of pseudotaxis, which is a form of translocation through the agar that does not result from chemotaxis signaling; these have been reported under similar conditions in several bacterial species (21–23). The aerotaxis defects of the ΔcheA4 and ΔcheY4 mutants could be complemented by expressing a parental gene from a broad-host-range plasmid but not by expressing a variant allele of cheA4 or cheY4 carrying a single mutation on the predicted phosphorylatable histidine (H54Q in CheA4) and aspartate (D57N in CheY4) residues, respectively (Fig. 2C and D). These data indicate that conserved phosphorylatable residues on CheY4 and CheA4 must be present for signaling to occur, suggesting that a phosphorylation cascade between CheA4 and CheY4 triggers changes in the direction of flagellar rotation to cause swimming reversals. Together, these results identify CheA4 to be the major histidine kinase mediating aerotaxis and chemotaxis in A. brasilense, and they further suggest that signaling from Che4 plays a major role in controlling the changes in the direction of flagellar rotation during swimming reversals.

The signaling output from Che4 is the control of the swimming reversal frequency. As indicated above, the formation of chemotaxis rings in the soft agar assay and the formation of aerotaxis bands in the capillary assay are not identical behaviors. The ultimate signaling output of a bacterial chemotaxis pathway is the control of the swimming motility pattern by affecting the probability of reversals. In other words, mutants unable to chemotax are expected to either constantly run or constantly reverse swimming direction. We assessed the swimming motility patterns of the Δche4, the ΔcheA4, and the ΔcheY4 mutants under steady-state conditions (Fig. 3A). The wild-type strain swam with long runs interrupted by instances of reversals that occurred with an average probability of about 0.5 reversals/s (Fig. 3B). In contrast to the wild type, mutants lacking CheA4 swam in straight runs and did not reverse (Fig. 3A and B). The ΔcheY4 mutant had a phenotype surprisingly different from that of the ΔcheA4 mutant, in that it also swam with fewer instances of reversals than the wild-type strain (Fig. 3A and B), but it clearly was still able to reverse swimming direction (Fig. 3A). The strain lacking the che4 cluster (Δche4) displayed a frequency of reversals that was not significantly different from that of the wild-type strain (0.6 reversal/s) (Fig. 3A and B). Analysis of free-swimming cells of the Δche4

FIG 3 Swimming behavior of the A. brasilense wild type and mutant derivatives lacking che4 genes or lacking a combination of che4 and che1 genes. (A) Tracks of free-swimming cells of A. brasilense and its che4 mutant derivatives. The tracks were obtained from digital recordings and computerized motion analysis using CellTrak software. Representative tracks are shown. Arrows, instances of swimming reversals. (B) Reversals were determined by computerized motion analysis from tracks of at least 75 free-swimming cells from instances of swimming reversals. (C) Tracks of free-swimming cells of A. brasilense and its derivatives lacking both che1 and che4 genes. The tracks were obtained from digital recordings and computerized motion analysis (CellTrak). Representative tracks are shown. Arrows, instances of swimming reversals.
strain also revealed a significantly erratic (jiggly) motility pattern (Fig. 3A). This analysis thus confirms that CheA4 is essential for the cells to reverse swimming direction. The lack of CheY4 or Che4 severely impaired but did not eliminate the ability of cells to reverse, while a lack of CheA4 completely abolished it, indicating that CheY4 (and, thus, Che4) has a major but not unique role in this behavior.

Che1 and Che4 together contribute to regulating the swimming pattern. The distinct swimming pattern of the ΔcheY4 and Δche4 strains compared to that of the ΔcheA4 strain was unexpected, since all three strains should display the same phenotype if the signaling output of the Che4 pathway is the control of swimming reversals. Che1 controls changes in swimming speed during chemotaxis, and previous data have suggested that signaling from Che1 and from the pathway affecting swimming reversals functionally interacted (6, 24). Therefore, we hypothesized that Che1 may be responsible for the residual swimming reversal ability of the ΔcheY4 and Δche4 mutants. To test this possibility, we constructed strains lacking both che1 and che4 (Δche1 Δche4; strain AB143), both cheA1 and cheA4 (ΔcheA1 ΔcheA4; strain AB141), and both cheY1 and cheY4 (ΔcheY1 ΔcheY4; strain AB142) (Table 1) and analyzed their swimming patterns (Fig. 3C). We found that the mutation of cheY1 and che1 in the ΔcheY4 and Δche4 mutant backgrounds yielded cells unable to reverse swimming direction, similar to the motility pattern observed for the ΔcheA4 strain or, as expected, for the ΔcheA1 ΔcheA4 strain (Fig. 3B and C). The disabling of Che1 in the Δche4 mutant (strain AB143) background also abolished the erratic swimming behavior of the Δche4 strain, suggesting that signaling from Che1 caused this behavior (Fig. 3B and C). The Δche1 Δche4 mutant strain and, to a lesser extent, the ΔcheA1 ΔcheA4 strain also persisted and swam in circles close to the surface of the coverslip (Fig. 3C). This behavior has been associated with smooth swimming close to surfaces in E. coli (25, 26) and is thus consistent with the lack of swimming reversals in these strains. However, we do not know why this behavior is more preeminent in these two mutants but not in the ΔcheY1 ΔcheY4 strain. Since Che1 affects the swimming speed, we also analyzed this motility parameter for mutants lacking che4 genes alone or in combination with mutations in che1 genes. We found that only the ΔcheA4 and the Δche4 mutants had a small, but significant, reduction in steady-state swimming speed compared to that of the wild-type strain (Fig. 4). This phenotype was absent in the ΔcheA1 ΔcheA4 and Δche1 Δche4 strains, implicating Che1 in the reduced swimming speed. These results suggest that it is the combination of reduced swimming speed and maintenance of the ability to reverse that caused the erratic swimming pattern of the Δche4 mutant strain (Fig. 3A). Together, the data confirm the role of Che1 and Che4 in regulating the motility patterns of swimming cells and in chemotaxis. They also provide further support to the hypothesis of a functional signaling interaction between Che1 and Che4.

The aerotactic advantage of coordinated regulation of swimming speed and reversals. We wondered what could be the advantage for A. brasilense cells in using two distinct chemotaxis signaling systems regulating two different parameters of swimming motility to modulate taxis responses. Che1, via signaling through CheY1 (6), regulates transient increases in swimming speed. Che4, via signaling through CheY4, controls reversals, as shown here. We hypothesized that a transient increase in the swimming speed that would accompany a suppression of swimming reversals during the response to an attractant would enhance the net movement of cells in this gradient. To test this hypothesis, we compared aerotaxis in a spatial gradient assay of the wild-type strain with that of its ΔcheY1 and ΔcheY4 mutant derivatives in a time course experiment (Fig. 5). As expected, the ΔcheY4 strain did not form an aerotactic band, although the cells were fully motile. The wild-type strain started forming an aerotactic band after 90 s, and a stable aerotactic band was formed by 150 s. The initiation of the aerotactic band took almost twice as much time for the ΔcheY1 strain, which formed a stable aerotactic band at 300 s postinoculation in the capillary tubes. This delay in the formation of the aerotactic band by the ΔcheY1 cells relative to the time of formation for the wild-type strain was consistently reproducible. These results suggest that the control of swimming speed by Che1 enhances the response of A. brasilense cells to a gradient of a major attractant, oxygen.

Che4 controls plant association. The major role of Che4 in controlling chemotaxis and the swimming pattern suggested that it should also play a significant role in the ability of cells to colonize the roots of cereals, such as wheat, a common host plant for A.
brazilense. When inoculated alone onto sterile wheat plants, the wild type and its Δche4 mutant derivative were able to colonize sterile wheat roots (Fig. 6A), but the mutants did so at a relatively reduced level compared to that for the wild type. Given that chemotaxis provides bacteria with a competitive advantage, we hypothesized that the colonization defects of the mutants would become apparent in competition experiments against the wild-type strain and thus compared the competitive index of the Δche4 mutant with that of the wild-type strain when they were inoculated at a 1:1 ratio (Fig. 6B). The competition experiments showed that a lack of che4 significantly impaired the competitive ability of the strains to colonize the wheat root surfaces, confirming the major role of this pathway in the lifestyle of A. brazilense in the rhizosphere.

**DISCUSSION**

Experimental evidence shows that Che4 signaling controls swimming reversals in A. brazilense and that it is the major pathway for all taxis responses in this species. As expected from its major function in regulating chemotaxis responses, Che4 is essential for competitive root surface colonization in A. brazilense. The A. brazilense Che4 pathway is orthologous to the major pathway controlling all chemotaxis responses in Sinorhizobium meliloti (27, 28) and R. leguminosarum (23), while its homolog in A. brasilense (Che1) controls swimming speed (6). Furthermore, mutations in A. brasilense che1 did not affect the ability to colonize the surface of wheat roots, indicating that this pathway does not contribute to the rhizosphere lifestyle of this organism (19). Lateral gene transfer (LGT) is considered a major driving force in the evolution of prokaryotes, including the acquisition of functions for adaptation to new niches (29). The A. brasilense Che4 pathway was previously identified to be one of the functions acquired by LGT by the ancestors of Azospirillum spp. that were proposed to have contributed to the adaptation to the rhizosphere (10). The role for Che4 in root surface colonization characterized here is thus consistent with this hypothesis. Che4 is a representative of the F7 class of chemotaxis systems (3) that was shown to be enriched in rhizosphere bacterial communities relative to those found in soil (2). The F7 chemotaxis systems, such as A. brasilense Che4, may thus be advantageous in this environment. However, the specific competitive advantage(s) that signaling via such a chemotaxis pathway provides remains to be identified.

The acquisition of Che4 by LGT in the ancestor of Azospirillum spp. did not cause Che1 to lose its function in chemotaxis. Indeed, our results indicate that both Che1 and Che4 have a role in the regulation of the swimming pattern of motile A. brasilense cells and thus chemotaxis. However, the contribution of Che1 and Che4 to this behavior is significantly different. As shown previously (6) and in the present study, Che1 controls transient increases in swimming speed, while Che4 controls the probability of swimming reversals and plays a major role in all taxis responses. Our data indicate that signaling via Che4 controls most swimming reversals, with Che1 modulating this behavior via direct effects on speed. This is in contrast to the role of two chemotaxis pathways in R. sphaeroides, a bacterial species that has been extensively studied in this respect (30). Two chemotaxis systems, named CheOp2 and CheOp3, are implicated in the control of chemotaxis in motile R. sphaeroides, and the signaling output from both chemotaxis pathways controls stops in the rotation of flagellar motors (31, 32). Consistent with a single signaling output, inactivation of either CheOp2 or CheOp3 abolishes chemotaxis in R. sphaeroides. This is because both signaling pathways ultimately affect the activity of a common set of CheY response regulators that bind the flagellar motors to stop their rotation (33, 34). This is in contrast to the results obtained here for A. brasilense that suggest that the signaling output from both Che1 and Che4, likely mediated via CheY1 (6) and CheY4, respectively, alters different parameters of flagellar motor activity. The control of chemotaxis by two Che pathways in A. brasilense thus illustrates a distinct strategy by which the signaling input from multiple Che pathways regulates chemotaxis.

Several lines of experimental evidence also support the hypothesis of a signal integration via a functional interaction between Che1 and Che4. First, inactivation of cheA4 completely suppressed reversals, and it also caused the cells to swim at a reduced speed compared to that of cells of the wild-type strain (24, 35). Second, strains lacking the CheY4 or Che4 function were significantly impaired but not null for chemotaxis unless cheY1 or che1, respectively, was also deleted. The discrepancy between the swimming speed phenotype of the ΔcheA4 and ΔcheY4 strains indirectly supports the hypothesis of a functional interaction between Che1 and Che4 because a ΔcheA1 strain, but not a ΔcheY1 strain, also swam at a slower speed than the wild-type A. brasilense strain.
vantage for marine bacteria in the exploitation of transient sources that change rapidly in space and time (37). In contrast to heterogeneous ties (38). Oxygen may be particularly limiting in the rhizosphere, to be significant in the competitive environment of the rhizosphere. Environ Microbiol

Increased swimming speed during chemotaxis provides an advantage for marine bacteria in the exploitation of transient sources of nutrients in the oceans (36). Like the ocean, the soil is a heterogeneous environment characterized by a plethora of nutrient gradients that change rapidly in space and time (37). In contrast to the ocean, the soil is less limiting in many essential nutrients, and as a result, it harbors abundant and diverse microbial communities (38). Oxygen may be particularly limiting in the rhizosphere, where microbial cell densities and microbial activities are the greatest (39, 40), making oxygen a potential modulator of microbial activities and competition in this environment. Consistent with this hypothesis, a major chemotaxis receptor for sensing oxygen and other metabolism-related parameters is critical for the ability of A. brasilense to colonize the wheat root surface (41). The results obtained here suggest that A. brasilense derives an aerotactic advantage from increased swimming speed, which is expected to be significant in the competitive environment of the rhizosphere. The additive roles of two chemotaxis systems controlling distinct motility parameters, illustrated here by the A. brasilense Che1 and Che4 systems, suggest a potential benefit that the control of chemotaxis by two (or more) pathways provides motile bacteria in a competitive environment. Given the ubiquitous distribution of multiple chemotaxis pathways in the genomes of rhizosphere bacteria, we expect this strategy to be widespread.

ACKNOWLEDGMENTS

This research is supported by National Science Foundation grant NSF-MCB 1330344 (to G.A.) and by the National Natural Science Foundation of China (31370108) and One Hundred-Talent Plan of the Chinese Academy of Sciences (CAS) (to Z.X.).

Any opinions, findings, conclusions, or recommendations expressed in this material are those of the authors and do not necessarily reflect the views of the National Science Foundation.

FUNDING INFORMATION

This work, including the efforts of Zhi Hong Xie, was funded by National Natural Science Foundation of China (NSFC) (31370108). This work, including the efforts of Tanmay Mukherjee, Dhivya Kumar, Nathan Bursiss, Zhi Hong Xie, and Gladys Alexandre, was funded by National Science Foundation (NSF) (MCB-1330344).

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