The Azospirillum brasilense Che1 Chemotaxis Pathway Controls Swimming Velocity, Which Affects Transient Cell-to-Cell Clumping

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The Che1 chemotaxis-like pathway of Azospirillum brasilense contributes to chemotaxis and aerotaxis, and it has also been found to contribute to regulating changes in cell surface adhesive properties that affect the propensity of cells to clump and to flocculate. The exact contribution of Che1 to the control of chemotaxis and flocculation in A. brasilense remains poorly understood. Here, we show that Che1 affects reversible cell-to-cell clumping, a cellular behavior in which motile cells transiently interact by adhering to one another at their nonflagellated poles before swimming apart. Clumping precedes and is required for flocculation, and both processes appear to be independently regulated. The phenotypes of a ΔaerC receptor mutant and of mutant strains lacking cheA1, cheY1, cheB1, or cheR1 (alone or in combination) or with che1 deleted show that Che1 directly mediates changes in the flagellar swimming velocity and that this behavior directly modulates the transient nature of clumping. Our results also suggest that an additional receptor(s) and signaling pathway(s) are implicated in mediating other Che1-independent changes in clumping identified in the present study. Transient clumping precedes the transition to stable clump formation, which involves the production of specific extracellular polysaccharides (EPS); however, production of these clumping-specific EPS is not directly controlled by Che1 activity. Che1-dependent clumping may antagonize motility and prevent chemotaxis, thereby maintaining cells in a metabolically favorable niche.

The ability of bacteria to sense and adapt to changes within their environment is an essential survival strategy. At the molecular level, signal transduction pathways couple sensing of environmental changes with adaptive responses which includes modulation of gene expression, enzyme activities, or protein-protein interactions (34). Chemotaxis in Escherichia coli is considered the best-studied signal transduction pathway. The E. coli chemotaxis signal transduction pathway functions to control the probability of changes in the flagellar motility pattern in response to physicochemical cues detected by dedicated chemotaxis receptors. These receptors form ternary signaling complexes with cytoplasmic chemotaxis proteins that include a histidine kinase, CheA, and an adaptor protein, CheW. Following repellent signal reception, CheA becomes autophosphorylated at a conserved histidine residue and phosphorylates its cognate response regulator, CheY. Phosphorylated CheY controls the probability of changes in the direction of flagellar rotation. The signaling activity of chemoreceptors is modulated by antagonistic activities of the methyltransferase CheR and the methylesterase CheB, thus allowing sensory adaptation. CheR constitutively adds methyl groups to specific glutamate residues in the C-terminal signaling regions of receptors, while CheB esterase activity depends on phosphorylated CheA. CheA is thus the central regulator of the chemotaxis response, as it links the forward excitation pathway that triggers the CheY-dependent signaling output with the feedback-adaptive loop that is dependent on CheB activity (28, 34). This prototypical chemotaxis signal transduction pathway is conserved in closely and distantly related bacterial species (36). Emerging evidence from the analysis of completely sequenced genomes indicates that most bacterial species possess more than one chemotaxis signal transduction pathway (30, 41). Additional chemotaxis-like signal transduction pathways (also named chemosensory signal transduction pathways) have been shown to be implicated in the regulation of nonmotility behaviors (15).

Azospirillum brasilense is an alphaproteobacterium and diazotrophic motile microorganism found in soil and rhizosphere habitats. A. brasilense has an oxidative metabolism that is optimum under microaerophilic conditions, with maximum energy generated at about 0.4% dissolved oxygen (40). Motile cells actively seek low oxygen concentrations for optimum metabolism by aerotaxis as well as by monitoring changes in the metabolic status via energy taxis (1, 3). Energy taxis is mediated by dedicated energy sensing receptors that allow A. brasilense to locate environments that are optimum for growth (1, 9, 40). Monitoring fluctuations in intracellular energy levels is the preeminent mode of sensing in A. brasilense (1), suggesting that in this organism, adaptive cellular behaviors such as aerotaxis are tightly coupled with metabolism.

The A. brasilense Che1 chemotaxis signal transduction pathway comprises homologs of CheA, CheW, CheY, CheB, and CheR that mediate energy taxis responses. Despite similarity to prototypical chemotaxis pathways, the A. brasilense Che1 pathway has been shown to be functionally divergent in that it appears to regulate taxis behaviors, as well as other cellular functions, including cell-to-cell clumping and flocculation (4). Experimental evidence indicates that che1 pathway mutants display changes in cell surface adhesive properties, likely in the structure and/or composition of extracellular polysaccharides (EPS), that ultimately modulate the ability of cells to clump and to flocculate (4, 7, 11, 27, 29). Furthermore, the contribution of Che1 to the control of motility-
dependent taxis responses seems to be more complex than those in other bacterial species, perhaps involving additional chemotaxis signal transduction pathways and/or auxiliary chemotaxis proteins (4, 29, 35). The Che1 pathway was also shown to indirectly affect changes in the swimming direction of cells, and thus the motility bias (4, 29). Prior biochemical and genetic evidence has suggested that CheB1 and CheR1 from the Che1 pathway participate in signaling crosstalk with another chemotaxis pathway(s) by altering the methylation status of chemoreceptors (29). In support of this possibility, the genome of A. brasilense encodes three chemotaxis-like signal transduction pathways, in addition to Che1, as well as several ancillary chemotaxis proteins (35). The functions of these chemotaxis-like pathways and proteins have not been determined.

In A. brasilense, flocculation results from the differentiation of motile, rod-shaped cells into aggregates of nonmotile, spherical cells that are encased in a dense fibrillar polysaccharide material (flocs) visible to the naked eye (25). Flocculated cells are not dormant but are highly resistant to various environmental insults (25). To date, the only known regulator affecting this differentiation process is an orphan transcriptional regulator, named FlcA, for which no cognate sensor kinase has been identified (13, 21, 22). Che1 signaling output might mediate changes in EPS production by directly affecting transcription of putative EPS biosynthetic genes. However, evidence for this hypothesis or in support of a genetic link between Che1 effects on taxis responses and on cell surface adhesive properties is lacking. In addition, the link between Che1 function and flocculation (and FlcA) remains un-investigated.

In this study, we clarify the role of Che1 in flocculation. By characterizing a set of che1 mutant strains and an energy taxis receptor mutant, we provide evidence that Che1 modulates reversible cell-to-cell interactions between motile cells, which we call “clumping.” We also show that Che1 does not directly regulate changes in EPS production. Interestingly, we found that the mechanism by which Che1 affects clumping behavior is by modulating the swimming velocity of cells. The role for Che1 in modulating transient cell-to-cell clumping via direct regulation of the swimming velocity provides insight into how this chemotaxis pathway may function to coordinate taxis behaviors with reversible cell-to-cell interactions in clumping.

MATERIALS AND METHODS

Bacterial strains and growth conditions. Table 1 describes the strains and plasmids used in this study. Cells were grown at 28°C with shaking in rich TY medium (1 liter contains 10 g tryptone and 5 g yeast extract) or MMB (minimal medium) (11). In order to induce clumping behavior and flocculation, 100 μl of an overnight culture of cells grown in TY medium (optical density at 600 nm (OD600) = 1) was used to inoculate 5 ml of flocculation medium (MMB containing 0.5 mM sodium nitrate as the sole nitrogen source and 8 mM fructose as the sole carbon source) and then grown with shaking at 28°C. For the time course of clumping and flocculation, cells were inoculated into flocculation medium and grown with high aeration and vigorous shaking (225 rpm). Aliquots of cultures were observed microscopically at the time of inoculation and at 3 h post-inoculation (hpi), 6 hpi, 9 hpi, 14 hpi, and 24 to 30 hpi (the time at which wild-type cells are flocculated). The times indicated in Table 2 were determined from multiple independent experiments (n = 5). Cells were visualized using a Nikon Eclipse E200 phase-contrast microscope at a final magnification of ×400, and photographs were taken of at least three different fields of view per sample per time point during this time course using a Nikon Coolpix P5000 camera. Digital videos were captured using a Sony Hyper HAD high-resolution black-and-white camera at a final magnification of ×400, and coverslips were added to enhance clarity. The timing of clumping and flocculation, as well as the accompanying behavioral and morphological changes observed, were highly reproducible.

Construction of a Δche1 flaC::Tn5 mutant (AB104) and of a ΔaerC Δche1 mutant (AB302). The Δche1 flaC double mutant strain was generated using the previously described pKmobGARc::cm construct, which was previously used to generate Δche1 (4). A biparental mating with the flaC::Tn5 mutant strain (Sp72002) as a recipient was performed as previously described (29) to construct the double mutant. Candidate Δche1 flaC (AB104) mutants were verified using PCR. The same technique was used to introduce a che1 mutation in the previously constructed ΔaerC Δche1 strain AB301 and generate the ΔaerC Δche1 strain AB302.

Complementation and site-specific mutations of che1 genes. Generation of constructs for complementation of Δche1 (AB101) was performed previously (4). The site-specific mutation cheA1H252Q was generated previously (unpublished data) using the QuikChange II site-directed mutagenesis kit (Stratagene). The following primers were used to generate cheA1H252Q; forward primer 5'-CATCTTCTCCCTGTTGAGACACATCGACAAAGGGACAC C3' and reverse primer 5'-GGTGCCCTTGTAGTGTTGCTGACCAGAGGAAAGATG C3'- (underlined letters refer to the specific bases which were mutated to change CAC [codon for histidine] to CAG [codon for glutamine]). cheA1H252Q was then cloned into pProEx-HTA (yielding plZ105). In order to generate pBBR-che1H252Q from its own promoter in the pBBR-MCS3 vector, the promoter region of the che1 operon, found upstream of cheA1, was first amplified from pBBR-cheA1 (4) using cheA1promXho-F (5'-CCGCTCGAGCGGGCAATGAACTG) and cheA1promXho-R (5'-AAGCTTACGGCAAGCTGACGAC) with the HindIII restriction site underlined and cheA1HQuo-R (5'-AACGTCGCCGGACATCCGAG GTT-3', with the XhoI restriction site underlined) and cheA1HQuo-R (5'-GCCCTTCTGAG CAGAAATCCC-3', with the HindIII restriction site underlined) and cheA1HQuo-R (5'-GCTTCGAATGATCGGACACCTTCTGAG C3'-, with the Xbal restriction site underlined) were used to amplify cheA1H252Q from plZ105. Each PCR fragment was cloned into the pcR2.1 TOPO vector and sequenced prior to cloning into pBBR-MCS3 (16). These fragments were inserted into the pBBR-MCS3 vector using the following digestions: cheA1HQuo (XhoI and HindIII), cheA1HQuo (HindIII and XbaI), and pBBR-MCS3 (XhoI and XbaI). The final product fuses the upstream region of cheA1 containing the putative promoter with cheA1H252Q in pBBR-MCS3. The final construct was introduced into wild-type A. brasilense Sp7 and the Δche1 strain (AB101) strain using biparental mating as described previously (29).

Constructs for complementation of Δchey1 (AB102) were also generated previously (4). The site-specific mutation cheY1D52N was generated by amplifying cheY1D52N from plZ103 (2), using the primers used to amplify cheY1 for complementation (4), and subcloning into the pcR2.1 TOPO vector. Upon verification by sequencing, cheY1D52N was isolated from this vector using restriction digestion with HindIII and EcoRI and ligated into pRK415 digested with the same enzymes (14). The newly constructed pRK-chey1D52N was introduced into the wild-type Sp7 strain and the Δchey1 mutant strain by biparental mating as described previously (29).

Complementation of the ΔcheB1 mutant was performed by amplifying the cheB1 gene from genomic DNA using the primers cheB1R1forward (5'- CCAAGCTTTAAGGAGAGCCCCCTAGTGGTCTGCTGCTGCTGCAGCA GAC-3', with the HindIII site underlined and the ribosome binding site in bold) (4) and cheB1R1forward (5'-GGGGCTCGAGCTCATCGCTGCCTGCTGCTGCTGCAGCA GAC-3', with the XbaI site underlined). The amplified cheB1 gene was subcloned into pcR2.1 TOPO and verified by sequencing. The cheB1 gene was isolated by digestion with HindIII and XhoI and ligated into pBBR-MCS3 digested with the same enzymes (16). The newly constructed pBBR-chB1 was introduced into the wild-type Sp7 strain and the Δchey1 (GA3) strain by biparental mating.

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The site-specific mutation cheB1\textsubscript{D78N} was generated using the QuikChange II site-directed mutagenesis kit (Stratagene) according to the manufacturer’s instructions with mutagenic primers CheBDN-F (5’-CGTCATCGTTCTCAACATCGAGATGCCGGTG-3’) and CheBDN-R (5’-CACCGGCACTCTCGATGTTGAGAACGATGACGTC-3’) (underlined letters refer to the specific bases which were mutated to change GAC [codon for aspartic acid] to AAC [codon for asparagine]) and cheB1 sub-cloned into pCR 2.1 TOPO as a template using cheB1R1forward and cheB1reverse (see above). The fragment generated (cheB1\textsubscript{D78N}) was cloned into pCR 2.1 TOPO, and the presence of the correct mutation was controlled (19). First, compressed air (21% oxygen) was allowed to flow over the cell suspension until cells are equilibrated (usually 5 min) before recording of the cells’ behavior begins. After 1 min, a valve is switched to provide pure nitrogen to the atmosphere of the cell suspension (air removal). The cells remain motile under these conditions. After a period of 6 min, the nitrogen gas flowing into the chamber is switched back to air (air addition), and recording is stopped after 3 min (10 min recorded total). Each experiment was performed at least 3 times on independent samples, and the behavior of the cells was highly reproducible.

The fraction of clumps in the cell suspension was determined as the number of clumps (rather than cells within clumps) relative to free-swimming cells. These ratios were measured using digital movies and the Cell Counter plug-in from ImageJ (http://rsbweb.nih.gov/ij/). Briefly, clumps were identified as transient cell-to-cell contacts between motile cells in suspension and counted manually for a given video frame or image. A minimum of 2 video frames were analyzed for each time point, and each video frame comprised at least 100 cells in the field of view. Clumping in samples, and the behavior of the cells was highly reproducible.

TABLE 1 Strains and plasmids used in this study

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Genotype or relevant characteristics(^a)</th>
<th>Reference or source</th>
</tr>
</thead>
<tbody>
<tr>
<td>pBBR1MCS3</td>
<td>Cloning vector (Tc)</td>
<td>16</td>
</tr>
<tr>
<td>pRK415</td>
<td>Cloning vector (Tc)</td>
<td>14</td>
</tr>
<tr>
<td>pBBR-cheA1</td>
<td>pBBR1MCS3 containing cheA1</td>
<td>4</td>
</tr>
<tr>
<td>pBBR-cheA1\textsubscript{H252Q}</td>
<td>pBBR1MCS3 containing cheA1\textsubscript{H252Q}</td>
<td>This work</td>
</tr>
<tr>
<td>pBBR-cheB1</td>
<td>pBBR1MCS3 containing cheB1</td>
<td>This work</td>
</tr>
<tr>
<td>pBBR-cheB1\textsubscript{D78N}</td>
<td>pBBR1MCS3 containing cheB1\textsubscript{D78N}</td>
<td>This work</td>
</tr>
<tr>
<td>pRK-cheY1</td>
<td>pRK415 containing cheY1</td>
<td>4</td>
</tr>
<tr>
<td>pRK-cheY1\textsubscript{D52N}</td>
<td>pRK415 containing cheY1\textsubscript{D52N}</td>
<td>This work</td>
</tr>
</tbody>
</table>

\(^a\) Antibiotic resistance abbreviations: Tc, tetracycline; Km, kanamycin; Gm, gentamicin; Cm, chloramphenicol.
point for each of the samples analyzed. The data shown are the averages of these calculations. In the temporal assay in the gas perfusion chamber, the fractions of clumps were measured at given time points during the assay before and after air removal or addition. The determined ratios were then plotted over time using Excel (Microsoft). The average swimming velocity of cells was determined on the same video files and frames described above, using computerized motion analysis (CellTrak 1.3; CellTrak, Santa Rosa, CA).

Extracellular complementation of clumping with extracted EPS. Extracellular polysaccharides (EPS) were extracted from 2-litter cultures of *A. brasilense* that were grown until clumping was observed. Samples of extracted EPS were used to treat 2 ml of wild-type cell suspension grown under flocculation-permissive conditions (but prior to clump formation), gently pelleted by centrifugation (2,500 × g for 2 min), and washed twice with 0.8% (wt/vol) sterile KCl in order to remove any EPS already present on the surface. We used this strategy because preliminary control experiments had shown that EPS present on the cell surface may mask the ability of exogenously added EPS to induce clumping. The cells were then resuspended in 100 μl of flocculation medium (see “Bacterial strains and growth conditions” above) with 100 μl of extracted EPS (at a final concentration of 15 μg/ml), followed by 1 to 2 h of incubation at room temperature with shaking. A control was incubated with water alone. Treated cells were then visualized using light microscopy. Photographs and digital videos were of cells treated with extracted EPS (or water as a control). The fraction of clumps was determined using both photographs and digital videos and manually counting the number of clumps (counting clumps rather than cells within clumps) relative to the total number of free-swimming cells in each field of view, with at least 3 distinct fields of view being analyzed per treatment and with counting of a total of at least 100 cells per sample. The fold increase in the clumping fraction was calculated relative to that of the control treated with water, which was taken as a reference and given the value of 1.0.

To test the effect of proteinase K treatment on the propensity of extracted EPS to mediate clumping, wild-type *A. brasilense* cells were grown under flocculation conditions and collected at the onset of clumping. The cells were then concentrated by centrifugation at 2,500 × g for 2 min to a final volume of 100 μl. The control samples remained untreated. Test samples were treated using (i) 100 μl of untreated wild-type EPS and (ii) 100 μl of proteinase K-treated EPS. The EPS fraction was analyzed on an SDS-polyacrylamide gel to verify the absence of protein after treatment with proteinase K. Cells were incubated for approximately 3 to 4 h at room temperature with shaking before visualization using light microscopy. The motility or viability of cells did not appear to be affected by these treatments.

Western blotting. In order to verify expression of CheA1 and CheY1 in the complementation constructs, we grew overnight cultures for each strain and then collected and lysed the cells. For samples from CheA1 complementation constructs, 250 ml of actively growing cultures was collected and cells were lysed by sonication in a lysis buffer containing 50 mM KH2PO4, 2 mM MgCl2, and 1 mM phenylmethylsulfonyl fluoride (PMSF) (a serine protease inhibitor). The lysate was then centrifuged for 5 min at 20,000 × g and 4°C, and the supernatant was removed for analysis by SDS-PAGE. Protein concentrations were measured for each sample using the Bradford assay (5), and each sample was adjusted to the same protein concentration. Next, a fresh solution of anti-CheY1 antibody (against CheY1 from *A. brasilense*) at 1:2,000 dilution, and an HRP-tagged anti-guinea pig secondary antibody at a 1:10,000 dilution. Analysis of CheB1 expression from the complementation constructs was not assessed due to the unavailability of an antibody against CheB1.

**RESULTS**

Che1 affects clumping but not flocculation. We have shown previously that mutants carrying deletions in cheA1, cheB1, cheY1, and che1 flocculate more than wild-type cells (4). We also found that a double mutant lacking both cheB1 and cheR1 flocculated very little, if at all (4). These data suggested that the Che1 pathway directly regulates flocculation. To gain further insight into how the Che1 pathway regulates the amount of flocculation, we observed the behaviors of each strain during this process. Cells were inoculated into flocculation medium (low nitrogen and high aeration) (see Materials and Methods), and the behaviors of the cells were analyzed at different times postinoculation (Fig. 1). We found that the strains carrying mutations within che1 differed not only in the amount of flocculation but also in the time at which they formed flocs. The ΔcheA1 mutant flocculated after 17 h of growth in the flocculation medium, as opposed to 24 to 30 h for the wild type (Fig. 1). The ΔcheB1 mutant flocculated the earliest, at 11 h postinoculation. The ΔcheY1 mutant also flocculated earlier than the wild type and at a time postinoculation that was intermediate between that observed for the ΔcheA1 and ΔcheB1 mutant strains, in that it flocculated 14 h postinoculation. Deletion of the che1 operon resulted in a strain (Δche1 mutant) that flocculated slightly earlier than the wild type, at 19 h. Consistent with previous data (4), the ΔcheB1cheR1 mutant did not flocculate (4). Finally, the ΔcheR1 mutant resembled the wild type, flocculating after 24 to 30 h. Therefore, the che1 mutant strains that flocculate more than the wild type initiate flocculation earlier than the wild-type strain.

Interestingly, when cells were grown under these conditions of flocculation, we observed that there was a difference not only in the amount and timing of flocculation between the strains but also in the amount and timing of clumping behavior. Free-swimming, wild-type cells formed small, transient, and highly dynamic “clumps” of two to five cells after approximately 9 h of incubation in the flocculation medium. These clumps consisted of cells that attached briefly (for about 1 s) at the cell poles before swimming apart (see Movie S1 in the supplemental material). Starting at around 15 h postinoculation, most cells appeared to be irreversibly attached to one another in larger clumps, and at about 18 to 19 h
postinoculation, larger aggregates comprised of nonmotile, round cells ("miniflocs") were observed. Cells within miniflocs appeared to be more refractile, suggesting that these cells contained intracellular polyhydroxybutyrate (PHB) granules (Fig. 1), an assumption confirmed by Nile red staining (data not shown). Over the next several hours, the miniflocs continued to grow larger by aggregation until the culture consisted primarily of large flocs (at 24 to 30 h postinoculation) (Fig. 1).

We observed that the ΔcheA1 mutant began to form small, stable clumps after approximately 3 h, much earlier than the wild-type strain (Table 2). The ΔcheB1 mutant showed clumping behavior resembling that of the ΔcheA1 mutant. The ΔcheY1 mutant formed clumps at 6 h postinoculation, which was earlier than the wild type but later than the ΔcheA1 mutant. Clumps formed by the ΔcheY1 mutant also appeared to be more transient initially than those produced by the ΔcheA1 and ΔcheB1 mutants. Clumping behavior was not observed in the Δ(cheB1 cheR1) mutant, while the ΔcheR1 mutant resembled the wild type. Differences in the timing of clumping behavior suggested that the effects of Che1 on flocculation instead reflect an effect of Che1 on clumping.

Formation of clumps and flocs earlier than for the wild type could explain why some Che1 mutants were shown previously to flocculate more than the wild type (4). However, the Δche1 mutant does not form clumps earlier than the wild type and flocculates only slightly earlier, making it difficult to explain why this mutant would flocculate more than the wild type. In order to gain insight into the mechanism by which the Δche1 mutant flocculates more than the wild type, we analyzed the amount of clumps (expressed as the fraction of clumps within the cell suspension) formed at different times postinoculation into the flocculation medium (Table 3). First, we compared the fractions of clumps in strains grown under flocculation-permissive conditions for 9 to 10
TABLE 2 Time course of clumping and flocculation in wild-type and mutant derivatives of A. brasilense

<table>
<thead>
<tr>
<th>Strain</th>
<th>Time (h) to:</th>
<th>Initiation of transient clumping</th>
<th>Transition to stable clumping</th>
<th>Flocculation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sp7</td>
<td>9 ± 1</td>
<td>11 ± 1</td>
<td>24 ± 3</td>
<td></td>
</tr>
<tr>
<td>AB101 (ΔcheA1)</td>
<td>2 ± 1</td>
<td>3 ± 2</td>
<td>17 ± 1</td>
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</tr>
<tr>
<td>GA3 (ΔcheB1)</td>
<td>2 ± 1</td>
<td>3 ± 2</td>
<td>11 ± 1</td>
<td></td>
</tr>
<tr>
<td>AB102 (ΔcheY1)</td>
<td>6 ± 2</td>
<td>9 ± 1</td>
<td>14 ± 1</td>
<td></td>
</tr>
<tr>
<td>AB103 (Δche1)</td>
<td>9 ± 1</td>
<td>11 ± 1</td>
<td>19 ± 2</td>
<td></td>
</tr>
<tr>
<td>BS109 (ΔcheR1)</td>
<td>9 ± 1</td>
<td>11 ± 1</td>
<td>24 ± 3</td>
<td></td>
</tr>
<tr>
<td>BS104 [Δ(cheB1 cheR1)]</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td></td>
</tr>
<tr>
<td>Sp72002 (flcA::Tn5)</td>
<td>9 ± 1</td>
<td>11 ± 1</td>
<td>—</td>
<td></td>
</tr>
<tr>
<td>AB104 (Δche1 flcA::Tn5)</td>
<td>9 ± 1</td>
<td>13 ± 1</td>
<td>—</td>
<td></td>
</tr>
<tr>
<td>AB301 (ΔaerC)</td>
<td>2 ± 1</td>
<td>3 ± 2</td>
<td>14 ± 1</td>
<td></td>
</tr>
<tr>
<td>AB302 (ΔaerCΔche1)</td>
<td>9 ± 1</td>
<td>11 ± 1</td>
<td>19 ± 2</td>
<td></td>
</tr>
</tbody>
</table>

aValues represent the time (mean ± standard deviation) postinoculation into flocculation media (n = 5); —, no clumping or no flocculation.

bTime at which transient cell-to-cell clumping is first detected. The fraction of transient clumps increases over time.

cTime at which stable cell-to-cell clumping is first detected. The fraction of stable clumps increases over time.

The time course analysis of clumping and flocculation revealed that clumping was initially a dynamic and reversible process. Over time, clumps became more stable, with motile cells losing the ability to leave clumps in order to return to a free-swimming behavior. Formation of these stable clumps could be a result of changes in EPS or in the production of specific "adhesins." A role for EPS in mediating clumping and flocculation in A. brasilense has been previously suggested (4, 27), prompting us to test this hypothesis. The EPS were extracted at about 10 h postinoculation into the flocculation medium, which corresponds to the time when wild-type cells have transitioned to formation of more stable clumps (Table 2). Under these conditions, cultures of the wild type and the ΔcheA1 and the flcA::Tn5 mutant strains displayed a significant proportion of stable clumps (Table 2). In contrast, cultures of the Δche1 and ΔcheY1 mutant strains were comprised mostly of cells displaying a transient clumping behavior (Table 2). As expected, clumping was not observed in cultures of the ΔcheB1 cheR1 mutant. Extracted EPS samples were then added exogenously at similar concentrations to a suspension of wild-type cells grown under flocculation-permissive conditions. EPS extracted from the wild-type, ΔcheA1, and flcA::Tn5 strains were able to induce stable clumping behavior per se (and not flocculation) in wild-type cells under these conditions; i.e., motile cells adhering to each other by their nonflagellated pales were observed in the EPS-treated samples but not in the control (Fig. 2A). Conversely, treatment of cells with EPS extracted from the Δ(cheB1 cheR1) strain, which does not exhibit any clumping behavior, was not able to induce clumping and in fact appeared to promote free swimming (Fig. 2A and B). Noticeably, the exogenous addition of EPS extracted from the ΔcheY1 and the Δche1 mutants failed to induce significant clumping under similar conditions (Fig. 2B). Exogenous application of EPS extracted from some, but not all, strains carrying mutations affecting Che1 function is thus sufficient to trigger the formation of stable clumps. EPS samples which were the most potent for inducing stable clumping behavior were extracted from strains that produced the most stable clumps at the time of EPS extraction: the wild-type strain and the ΔcheA1 and the flcA::Tn5 mutant strains. Strains that clumped only transiently at the time of EPS extraction (the ΔcheY1 and Δche1 mutants) produced EPS that did not trigger stable clumping. Therefore, it is likely that EPS production contributes to stabilizing clumping. However, mutations within different che1 genes appear to yield strains producing EPS of variable potency for inducing stable clumping, indicating some variation in the nature of the EPS produced by these strains. This observation supports the hypothesis of an indirect role for Che1 in modulating "clumping-specific" EPS production. To rule out any other indirect effects of the extracted EPS, the contribution of proteins potentially present within the extracted EPS was tested by examining the effect of proteinase K treatment of EPS on induc-
because clumping is observed when cultures of A. brasilense are grown under conditions of high aeration (4). Therefore, we modified a gas perfusion chamber assay, typically used for aerotaxis (1), to directly measure temporal changes in clumping in order to further characterize the transient and reversible clumping behavior. In this assay, the gas atmosphere flowing above a suspension of cells can be controlled, allowing one to analyze the behavioral responses of cells challenged with changes in the aeration conditions. In order to use the gas perfusion chamber assay to analyze clumping, we also grew all strains under high-aeration conditions and to higher cell densities (late exponential phase) (see Materials and Methods). Under these growth conditions, all strains clumped in a pattern similar to that detected in the flocculation assay; i.e., the ΔcheA1, ΔcheY1, Δche1, ΔcheB1, and ΔcheR1 mutant strains had more clumps than the wild type, while the Δ(cheB1 cheR1) mutant did not clump (Fig. 1). When a suspension of wild-type cells was analyzed in this assay, cells responded to a switching in the aeration (air removal) with a decrease in the overall amount of clumping (reduction by half relative to prestimulus levels) (Fig. 3) followed by an adaptation period where the fraction of clumps detected in the suspension returned to prestimulus levels. We observed that the fraction of clumps decreased transiently upon air removal because clumped cells briefly returned to free-swimming behavior before adapting to the ambient aeration conditions and returning to clumps (Fig. 3). Similarly, when air was returned to the atmosphere of the cell (air addition), cells displayed a similar response, characterized by a similar decrease in clumping by half concomitant with the clumped cells becoming free swimming for a short period before the clumps reformed to return to prestimulus levels (Fig. 3).

The ΔcheR1 mutant resembled wild-type cells the most (Fig. 3). The ΔcheR1 mutant differed from the wild type in that after the initial decrease in the clumping fraction (upon both air removal and air addition), cells were slower to return to prestimulus clumping levels. Whereas wild-type cells would begin to reform clumps after 30 to 40 s, the ΔcheR1 mutant would take approximately 60 s to resume clumping behavior. The Δ(cheB1 cheR1) mutant did not show any clumping behavior, regardless of the removal or addition of air (Fig. 3).

The clumping behavior of the ΔcheA1 mutant differed in two key ways from that of the wild-type strain, which responded to both air addition and air removal with an initial decrease in clumping behavior (prior to adaptation where clumps were reformed), in this assay. First, the ΔcheA1 mutant responded to both air removal and air addition, but it did not adapt to the imposed aeration conditions since clumping did not return to prestimulus levels upon air removal or air addition (Fig. 3). Second, rather than there being a decrease in the number of clumps upon air removal, clumping levels of this mutant in suspension nearly doubled under these conditions. Upon air addition to the ΔcheA1 cell suspension, the number of clumps decreased by half. Similar to the case for the ΔcheA1 mutant, the ΔcheB1 mutant also responded to both air removal and air addition, but no adaptation to the changes in aeration conditions was observed. The number of clumps in the suspension of the ΔcheB1 mutant also doubled upon air removal and decreased upon air addition by the same amount, as for the ΔcheA1 mutant.

In response to air addition, clumping also nearly doubled in the suspension of the ΔcheY1 mutant, which contrasted with the wild-type response to similar changes in aeration conditions. In fact, the observed increase in clumping seen in the ΔcheY1 mutant upon air removal is similar to that observed in the ΔcheA1 and ΔcheB1 mutants; however, clumping in the ΔcheY1 mutant slowly adapted to the new aeration conditions and decreased back to prestimulus levels (Fig. 3). No further change in clumping was detected when air was returned to the atmosphere of the ΔcheY1 mutant (air addition). The Δche1 mutant increased clumping by more than 2-fold, which is slightly more than that observed in the other mutants; however, like for the ΔcheY1 mutant, clumping in response to air removal slowly adapted and returned to prestimulus levels (Fig. 3). Similar to the case for the ΔcheY1 mutant, the

![A. brasilense](http://jb.asm.org/content/194/13/3349/F2.large.jpg)

**FIG 2** Effects of extracted exopolysaccharides (EPS) on clumping in Azospirillum brasilense. (A) Representative light microscopy photographs of wild-type cells treated with EPS extracted from the strains indicated at the top. Arrows indicate clumping cells. (B) Fold increase in clumping upon treatment of wild-type cells with EPS extracted from different strains. The fold increase in the clumping fraction upon treatment with the extracted EPS compared to the control treatment (water), taken as a value of 1, is shown. (C) Effect of protease K on the ability of extracted EPS to induce clumping. A suspension of wild-type A. brasilense cells was prepared as for panel A and then treated as indicated. Arrows indicate clumping cells. The data shown represent at least three independent experiments.
A che1 mutant did not respond to air addition, and no change in clumping was detected.

Collectively, our results show that changes in transient clumping take place as a result of changes in aeration conditions. These results also indicate that Che1 functions to reduce clumping when cells experience a decrease in aeration (air removal), but its contribution to clumping under conditions of increased aeration (air addition) is not straightforward.

Clumping is not correlated with changes in reversal frequency. Changes in the clumping pattern of wild-type cells detected in the gas perfusion chamber assay were reminiscent of changes in the swimming pattern of cells in a temporal assay for chemotaxis or aerotaxis, suggesting a functional link between clumping and motility. First, we analyzed the swimming motility bias of cells that have experienced changes in clumping upon changes in aeration conditions in the gas perfusion chamber assay described above. Under these conditions, the wild-type strain was found to display a nearly 3-fold decrease in the swimming reversal frequency (number of reversals in the swimming direction per second and per cell) upon air removal from or addition to the atmosphere of the cells (see Fig. S2 in the supplemental material). While most of the che1 mutant strains responded to changes in aeration conditions by modulating the swimming motility bias under these conditions, no correlation between changes in clumping and changes in the reversal frequency of cells upon air removal or air addition could be established when analyzed in this assay (see Fig. S2 in the supplemental material). The most striking illustration of this fact is that no change in the swimming bias of the ΔcheR1 mutant strain could be detected upon air removal or addition, yet its clumping behavior most resembled that of the wild-type strain, albeit with longer transient free-swimming responses (Fig. 3; see Fig. S2 in the supplemental material).

Clumping and swimming velocity correlate. While there was no detectable correlation between clumping behavior and changes in the motility bias, changes in swimming velocity appeared to correlate with changes in clumping detected upon air removal. For example, wild-type cells showed a significant increase in swimming velocity of approximately 3 μm/s upon air removal,
which correlated with a transient decrease in the fraction of clumping cells by about half (Fig. 3). When the swimming velocity of wild-type cells decreased to prestimulus levels upon adaptation, the clumping fraction increased to prestimulus levels as well. Similar to the case for the wild-type strain, the ΔcheR1 mutant cells also swam faster, by about 5 μm/s, in response to air removal, with a concomitant decrease in clumping by half. The Δ(cheB1 cheR1) strain, on the other hand, did not respond to air removal by modulating clumping, but it did respond by transiently swimming faster (a ~5-μm/s increase in swimming velocity). Notably, the average swimming velocity of the Δ(cheB1 cheR1) mutant strain was also the highest of all strains analyzed (ranging from 30 to 37 μm/s), differing from that of the wild type by about 4 μm/s and from that of the ΔcheA1 mutant (the slowest of the Che1 mutants) by nearly 14 μm/s (Fig. 3). Given that clumping decreases with increased swimming velocities in the wild type, the higher average swimming velocity of the Δ(cheB1 cheR1) mutant may explain the lack of clumping observed in this strain (Fig. 3). In contrast, the swimming velocities of the mutants lacking cheA1, cheB1, cheY1, or che1 decreased by an average of 3 to 7 μm/s upon air removal, and this was concomitant with a doubling in the amount of clumping in cell suspensions of these mutants (Fig. 3). These results thus suggest that the inability of these che1 strains to increase swimming velocity upon air removal correlates with the increase in clumping observed under these conditions (Fig. 3). These data further imply that changes in transient clumping upon air removal may be modulated by direct effects of Che1 on the swimming velocity.

Although changes in swimming velocity correlated with changes in clumping upon air removal in the che1 mutant strains, we were unable to detect such a correlation upon air addition (Fig. 3). Changes in swimming velocity detected for the wild type and the various che1 mutant strains upon air removal were also detected and followed a similar pattern upon air removal (Fig. 3). While the wild-type cells as well as the Δche1 and Δ(cheB1 cheR1) mutant cells swam transiently faster by about 5 μm/s upon air addition, the ΔcheA1, ΔcheB1, ΔcheY1, and Δche1 mutant cells swam with a reduced velocity (by 4 μm/s on average) under these conditions. Similar to the behavior observed upon air removal, the transient increase in the swimming velocity correlated with a decrease in clumping by half for the wild type and the ΔcheR1 mutant, while clumping was not detected for the Δ(cheB1 cheR1), mutant for which the average swimming velocity remained the greatest (Fig. 3). In contrast and despite transiently slower swimming, clumping decreased by half in suspensions of the ΔcheA1 and the ΔcheB1 mutant strains upon air addition, while there was no change in clumping (no response) in suspensions of the ΔcheY1 and Δche1 mutants (Fig. 3). These observations suggest that the effect of the decreased swimming velocity on clumping may be significant only when cells experience a decrease in aeration conditions (air removal). This suggests that transient clumping is modulated by direct effects of Che1 on the swimming behavior, as well as by other Che1-independent effects that are likely prevalent under conditions of increased aeration in the atmosphere of the cells.

Control of the swimming velocity is a signaling output of Che1. In order to gain further insight into how signaling via Che1 regulates the swimming velocity and clumping, the behavior of mutant strains expressing wild-type che1 genes or genes carrying mutations of conserved phosphorylation residues expressed from low-copy-number plasmids was characterized under flocculation conditions. The swimming velocities of the mutants lacking cheA1, cheB1, cheY1, or che1 decreased by an average of 3 to 7 μm/s upon air removal, and this was concomitant with a doubling in the amount of clumping in cell suspensions of these mutants (Fig. 3). These results thus suggest that the inability of these che1 strains to increase swimming velocity upon air removal correlates with the increase in clumping observed under these conditions (Fig. 3). These data further imply that changes in transient clumping upon air removal may be modulated by direct effects of Che1 on the swimming velocity.

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conditions (see Table S1 in the supplemental material) and in the gas perfusion chamber assay (Fig. 4; see Fig. S3 in the supplemental material). Expression of CheA1, CheA1D252Q, CheY1, and CheY1D32N from the plasmids used for complementation was detected with antibodies to the A. brasilense wild-type proteins and found to be comparable to the level of the wild-type proteins (see Fig. S1 in the supplemental material). Similar controls of protein expression could not be performed with CheB1, since we do not have any antibody to CheB1 and none of the antibodies against CheB of other bacterial species that we have tried cross-reacted with the CheB1 protein from A. brasilense. While functional complementation of ΔcheA1 (AB102) with the wild-type cheA1 expressed from a plasmid restored wild-type clumping behavior in both the flocculation and the gas perfusion chamber assays, complementation with a plasmid expressing a mutated cheA1 gene in which the codon for the conserved phosphorylatable histidine residue is replaced with glutamine (CheA1H235Q) failed to restore the wild-type phenotype: the pattern of changes in clumping within the suspension upon air removal or addition paralleled that of the mutant strain (Fig. 4; see Fig. S3 in the supplemental material). An intact CheA1 is thus required for its function in clumping. Similar functional complementation of ΔcheY1 (AB102) with the wild-type CheY1, but not with a CheY1H352Q variant, restored the wild-type clumping phenotype, indicating that functional CheY1 is also essential for its function in clumping (Fig. 4; see Fig. S3 in the supplemental material). An intact phosphorylatable aspartate site (D78) in the receiver domain of CheB1 was found to be required to restore the wild-type clumping behavior of a ΔcheB1 mutant strain (see Fig. S3 in the supplemental material). Functional CheA1 and CheY1, but not CheA1H235Q or CheY1D32N, also restored the ability of strains to transiently increase swimming velocity upon air removal and air addition in the gas perfusion chamber assay, correlating with a transient reduction in the fraction of clumped cells (Fig. 4). Che1 thus modulates the cell’s swimming velocity by a direct effect on CheY1 phosphorylation, likely via a phosphoryl relay between CheA1 and CheY1. The analysis of the ΔcheA1, ΔcheY1, and Δche1 mutant phenotypes together with the effects of expressing nonfunctional CheA1H235Q or CheY1D32N on the swimming velocity of cells also indicates that Che1 signaling output causes an increase in the swimming velocity of cells.

A taxis receptor modulates Che1-dependent effects on clumping. Mutants with mutations affecting the A. brasilense energy taxis receptor aerC were previously shown to possess small clumps under most growth conditions (37), suggesting that this receptor functions to modulate clumping. Consistent with this hypothesis, a ΔaerC mutant strain clumped after 1 to 3 h postinoculation (Table 2) and did so quantitatively more than the wild-type strain, resulting in greater flocculation as well (data not shown). A double mutant lacking both aerC and che1 (ΔaerC Δche1, strain AB302) behaved like the Δche1 mutant strain (Table 2) for clumping and flocculation. This is consistent with previous observations (37) and suggests that AerC functions upstream of Che1 to modulate clumping.

Next, the behavior of the ΔaerC (AB301) and the ΔaerC Δche1 (AB302) mutant strains were analyzed in the gas perfusion chamber assay (Fig. 3). The swimming velocities of both the ΔaerC and the ΔaerC Δche1 mutant strains decreased by at least 5 μm/s immediately upon air removal. Upon air addition, the swimming velocity increased by approximately 5 μm/s in the ΔaerC strain, but decreased by 5 μm/s in the ΔaerC Δche1 strain. These results are consistent with the role of Che1 in modulating changes in swimming velocity as well as with the role of AerC in mediating Che1-dependent changes in the swimming velocity, likely by sensing conditions of decreased aeration.

Compared to the wild-type strain, the ΔaerC mutant strain doubled in clumping, but with a significant delay (of about 15 s compared to the result for the wild type) relative to the time of air removal. The delayed clumping response seen in the ΔaerC mutant strain upon air removal was no longer observed in the ΔaerC/Δche1 strain; clumping increased immediately upon air removal (Fig. 3). Similar to the case for the ΔcheA1 and ΔcheB1 mutants, clumping in each of these strains remained at levels higher than those under prestimulus conditions after it initially increased upon air removal. Clumping returned to lower levels only after air addition in the ΔaerC mutant strain (Fig. 3). Under conditions of increased aeration, the ΔaerC/Δche1 mutant strain responded by decreasing the fraction of clumps by more than 2-fold (Fig. 3). In contrast to the response of the ΔaerC mutant, the decrease in clumping upon air addition was transient in the ΔaerC/Δche1 strain (Fig. 3). The ability of the ΔaerC Δche1 mutant to respond to air addition by transiently decreasing clumping also lends support to the hypothesis that the effects of Che1 on the swimming speed do not directly contribute to clumping under conditions of increased aeration. The transiently decreased clumping response of the ΔaerC Δche1 mutant upon air addition is similar to the response of the wild-type strain, i.e., appears to correspond to adaptation; however, steady-state clumping of the wild-type strain in suspension remained at lower levels. In contrast, the steady-state levels of clumping remained at significantly higher levels relative to those prestimulus in suspension of the ΔaerC Δche1 mutant, suggesting indirect effects of AerC on the steady-state levels of clumping. Taken together, these results argue that in addition to modulating Che1-dependent effects on transient clumping via an effect on the swimming speed, AerC may also mediate indirect effects that modulate the ability of cells to maintain constant steady-state levels of clumping after changes in aeration conditions.

**DISCUSSION**

The Che1 chemotaxis-like pathway of A. brasilense has been implicated in the control of chemotaxis and aerotaxis as well as cell surface adhesive properties, clumping, and flocculation. Here, we show that clumping and flocculation are distinct processes and that Che1 contributes to the control of the initial reversible and transient cell-to-cell clumping behavior. While transient clumping correlates with changes in motility of cells, the transition to stable clumps depends on the production of EPS that are produced independently of Che1. Our results demonstrate that the signaling output of Che1 is the modulation of the flagellar swimming velocity, illuminating its role in chemotaxis and aerotaxis.

The signaling output of Che1 is the control of swimming velocity. Che1 appears to directly regulate the propensity of cells to increase swimming velocity with changes in aeration conditions. A functional Che1 pathway, including a phosphorylatable CheY1 response regulator, is required for this response, identifying modulation (increase) of the swimming velocity as a signaling output of the Che1 pathway. This finding implies that changes in swimming velocity contribute to aerotaxis in A. brasilense, with receptors signaling to Che1 upon changes in aeration conditions in
order to modulate this response. AerC is an energy taxis receptor (37) that has been shown here and elsewhere (37) to function in a Che1-dependent manner to modulate the swimming velocity and aerotaxis. Changes in swimming velocity have not been directly implicated in the tactic behaviors of *A. brasilense*. However, the results obtained here imply that changes in the swimming speed of cells represent a mechanism by which motile *A. brasilense* cells actively navigate in gradients. This assumption is supported by the observation that wild-type *A. brasilense* cells responded to temporal changes in aeration conditions by increasing the swimming speed, as well as decreasing the reversal frequency by nearly 3-fold (an attractant response). Further, the ∆(*cheB1 cheR1*) mutant strain, in which CheA1 and CheY1 are supposed to be active (5), has a constantly greater swimming velocity than the wild-type cells (Fig. 3). In addition to identifying the signaling output of the Che1 pathway, these results also shed light on how the Che1 pathway could contribute to chemotaxis and aerotaxis, while not having any apparent effect on the reversal frequency of the cells (4). Che1 signaling output could regulate swimming velocity either via a direct effect(s) of phosphorylated CheY1 on the flagellar motor or switch complex to ultimately increase flagellar swimming velocity or through indirect effects involving an additional protein(s) capable of affecting flagellar motor rotation or torque. Direct effects of CheY proteins on flagellar rotation have been recently demonstrated in *Rhodobacter sphaeroides*, a species that employs multiple CheY homologs which are able to bind to the flagellar motor and switch complex and that act as a “brake” to stop flagellar rotation (23). The genome of *A. brasilense* encodes six CheY homologs that have not been characterized yet (35). The role of CheY1 in increasing the swimming velocity and the contribution of Che1 to chemotaxis and aerotaxis in *A. brasilense*, albeit minor (4, 11), collectively suggest the possibility of a similar function. However, other mechanisms are possible. For example, CheY1 could counteract the effect of a potential flagellar velocity braking protein(s) and thus act indirectly to increase the speed at which cells swim. The observation that mutations affecting *cheA1* or *cheY1* or deletion of *cheI* causes the cells to respond to changes in aeration conditions by swimming more slowly than the wild type (Fig. 3) gives credence to this hypothesis. Several proteins from diverse bacteria have been recently shown to control the flagellar motor with typical effects observed on the cell's swimming velocity. The proteins involved and the mechanisms by which they interact with flagellar motor components appear to be diverse, even within a single bacterial species, making their direct identification from sequences alone challenging (for a recent review, see reference 6).

**Che1-dependent changes in swimming speed modulate transient clumping.** When wild-type cells were analyzed in a temporal assay for clumping, a transient decrease in the fraction of clumps was observed upon air removal from and air addition to the atmosphere of the cells. Concomitant with this transient change in cell-to-cell interactions, swimming was biased toward less-frequent changes in the swimming direction (reversals per second), and the swimming speed increased immediately in response to changes in aeration conditions. Changes in clumping paralleled changes in the swimming pattern (bias and velocity) of the cells. The results obtained here establish that changes in the swimming velocity mediated by Che1 are sufficient to promote a reduction in transient clumping upon downshifts in aeration conditions. Changes in the swimming speed could affect clumping, perhaps by increasing the likelihood of loosely adherent cells within clumps detaching and swimming away and/or by decreasing the probability of initial cell-to-cell contacts, thus reducing the possibility of transient clump formation. A decrease in flagellar swimming bias and/or flagellar swimming velocity was previously shown to promote surface attachment and biofilm formation in many bacterial species (8, 18, 19, 32). Similarly, changes in the swimming motility of *A. brasilense* cells appear to contribute to the propensity of cells to interact within clumps. A major role for Che1-dependent changes in motility in modulating cell-to-cell interactions in transient clumps is consistent with the dynamic and short-lived nature of these associations and with the propensity of transient clumps to dissociate rapidly in response to changes in aeration conditions. While Che1 appears to control the flagellar swimming velocity under all conditions, a significant effect of the swimming speed on transient clumping was detected only upon air removal, under the conditions of the gas perfusion chamber assay. This suggests that while the swimming velocity may significantly affect transient clumping under some conditions, another behavior(s) may modulate the dynamics of transient reversible clumping under other conditions, such as an increase in aeration. Given the changes in the motility pattern of the wild-type cells detected upon air removal and air addition, it is likely that the swimming reversal frequency or perhaps other transient changes in the motility behavior that were not detected here could contribute to modulate clumping upon increases in aeration. Given that clumping was correlated with increased biofilm formation in *A. brasilense* (27), these results also raise the intriguing possibility that changes in the swimming pattern of cells are directly implicated in the initiation of transient cell-to-cell and cell-to-surface interactions that precede most steps in colonization.

**Cross talk may be involved in modulating clumping.** In addition to direct effects on the swimming velocity, Che1 also appears to have indirect effects on clumping behavior. For instance, some Che1 mutants show increased steady-state levels of clumping that do not return to prestimulus levels in the gas perfusion chamber assay, which did not appear to directly correlate with a generally lower swimming velocity of the cells. Indirect effects also include the observation that *cheI* mutations affect the time to clumping in the flocculation assay, consistent with effects of Che1 on the sensitivity of the clumping response under the conditions of this assay. Adaptation proteins in chemotaxis signal transduction function to maintain response sensitivity over a broad range of background conditions and allow the behavior to return to prestimulus bias after a rapid response to environmental stimuli (15, 34). Loss of function in adaptation proteins is thus expected to affect the sensitivity and/or activity of chemotaxis receptors that are regulated by these proteins. The following lines of evidence support the hypothesis that indirect effects of Che1 on clumping are mediated through the activity of adaptation proteins and chemotaxis receptors. First, mutations affecting CheB1 directly or indirectly (e.g., mutations affecting CheA1 function) yielded the most dramatic clumping phenotypes. Second, increased clumping triggered in response to air removal from the atmosphere of the cells remained at high levels in strains with a ∆*cheA1* or ∆*cheB1* mutation or expressing nonfunctional CheA1 or CheB1 variant proteins. Persistent clumping at high levels was also detected in a mutant strain lacking a functional AerC receptor. This clumping response pattern suggested that a stimulus and an initial response
must be implemented for this change in the steady-state clumping behavior to be detected, implicating defects in a response feedback loop. In contrast, higher clumping levels decayed slowly to reach lower levels in strains carrying ΔcheY1 (or the nonfunctional CheY1D52N variant) and Δche1 mutations. Last, a strain carrying a Δ(cheB1 cheR1) mutation does not clump under any growth conditions. Collectively, these results implicate both a receptor (AerC) and Che1 adaptation proteins, in particular CheB1, in mediating the indirect effects of Che1 on the sensitivity of cells that allow them to maintain clumping at constant steady-state levels. How could indirect effects of Che1 be mediated by adaptation proteins and/or receptors? One possibility is that other pathways in addition to Che1 contribute to modulating the swimming behavior and thus transient clumping. The results obtained here and in previous studies (5, 29) support this hypothesis. CheB1 and CheR1 of the Che1 pathway were previously implicated in a signaling cross talk between Che1 and another, unknown pathway(s) in regulation of aero- and chemotaxis responses in A. brasilense, with the most notable effects being the relative sensitivity of receptors and the associated pathway(s) to changes in environmental conditions (29). Genetic, biochemical, and/or modeling data for a few other bacterial species support the hypothesis that cross talk between different chemotaxis or Che-like operons may be mediated by the activity of CheB proteins (10, 20, 24, 31, 33). The methylation status of the FrzCD receptor also depends on signaling by two Che-like pathways in Myxococcus xanthus (38), lending further support to this possibility. In such a scenario, if receptors are modified by adaptation proteins from independent signaling pathways, then sensory input to one pathway could modulate the activity of the adaptation protein of this pathway, which in turn will affect the sensitivity and thus the response output of another pathway(s).

Clumping and its relationship with flocculation. While they are both formed as a result of cell-to-cell interactions, clumping cells are morphologically and behaviorally distinct from flocculated cells in that flocculated cells are nonmotile, round, and encased in large aggregates of polysaccharides (25). Consistent with this observation, FlcA, a key transcriptional regulator for flocculation (21), has no effect on clumping in A. brasilense, with the most notable effects being the relative sensitivity of receptors and the associated pathway(s) to changes in environmental conditions (29). Genetic, biochemical, and/or modeling data for a few other bacterial species support the hypothesis that cross talk between different chemotaxis or Che-like operons may be mediated by the activity of CheB proteins (10, 20, 24, 31, 33). The methylation status of the FrzCD receptor also depends on signaling by two Che-like pathways in Myxococcus xanthus (38), lending further support to this possibility. In such a scenario, if receptors are modified by adaptation proteins from independent signaling pathways, then sensory input to one pathway could modulate the activity of the adaptation protein of this pathway, which in turn will affect the sensitivity and thus the response output of another pathway(s).

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