A Chemotaxis-Like Pathway of *Azorhizobium caulinodans* Controls Flagella-Driven Motility, Which Regulates Biofilm Formation, Exopolysaccharide Biosynthesis, and Competitive Nodulation

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The genome of the *Azorhizobium caulinodans* ORS571 contains a unique chemotaxis gene cluster (*che*) including five chemotaxis genes: *cheA*, *cheW*, *cheY*, *cheB*, and *cheR*. Analysis of the role of the chemotaxis cluster of *A. caulinodans* using deletion mutant strains revealed that CheA or the Che signaling pathway controls chemotaxis behavior and flagella-driven motility and plays important roles in formation of biofilms and production of extracellular polysaccharides (EPS). Furthermore, the deletion mutants (Δ *cheA* and Δ *cheA-R*) were defective in competitive adsorption and colonization on the root surface of host plants. In addition, a functional CheA or Che pathway promoted competitive nodulation on roots and stems. Interestingly, a nonflagellated mutant, Δ *fliM*, displayed a phenotype highly similar to that of the Δ *cheA* or Δ *cheA-R* mutant strains. These findings suggest that through controlling flagella-driven motility behavior, the chemotaxis signaling pathway in *A. caulinodans* coordinates biofilm formation, EPS, and competitive colonization and nodulation.

Nitrogen-fixing bacteria are generally divided into two major classes, free-living and symbiotic. Symbiosis between rhizobia and hosts generally leads to formation of nodules on roots. Some members of legumes are also able to develop stem nodules (James et al. 2001; Tsien et al. 1983). *Azorhizobium caulinodans* ORS571 has the capability of fixing nitrogen both in the free-living and under symbiotic conditions with its host

Sesbania rostrata (Dreyfus et al. 1983). In addition, it retains a capacity to induce nodule formation on roots as well as on stems of *S. rostrata* under symbiotic conditions (Dreyfus et al. 1983, 1988; Tsien et al. 1983).

The formation of biofilms on the root surface promotes colonization in many plant-associated bacteria, including genera such as *Rhizobium*, *Gluconacetobacter*, and *Azospirillum* (Rinaudi and Giordano 2010; Siuti et al. 2011; Yaryura et al. 2008). Furthermore, rhizobial extracellular polysaccharides (EPS) have been proposed to play important roles in adhesion and colonization of root surfaces (Burdman et al. 2000; Matthysse et al. 2005; Michiels et al. 1991; Santaella et al. 2008), formation of biofilms on roots during nodulation (Bianciotto et al. 2001; Frayssse et al. 2003; Fujishige et al. 2006; Meneses et al. 2011; Ramey et al. 2004; Rinaudi and Giordano 2010; Skorupska et al. 2006). Mutants with altered EPS are defective in symbiosis with host plants (Cheng and Walker 1998; Doherty et al. 1988; Wells et al. 2007). In soil bacteria, chemotaxis and motility favor the chemical or physical interaction with host roots and occupation of infection sites resulting in more competitive colonization and nodulation ability (Caetano-Anollés et al. 1988; Greer-Phillips et al. 2004; Miller et al. 2007; Van de Broek and Vanderleyden 1995).

Bacterial chemotaxis is a motility-based response initiated by perception of environmental chemical signals. The chemotaxis signal transduction pathway has been best studied in *Escherichia coli* (Wadhams and Armitage 2004). This pathway is comprised of conserved proteins that include a histidine kinase CheA, an adaptor protein CheW, a response regulator CheY, a methyl-esterase CheB, a methyltransferase CheR, and multiple chemoreceptors. CheA acts as the central processing unit of this system. Environmental signals sensed by chemoreceptors regulate the phosphorylated state of CheA via CheW. The phosphoryl group is then transferred from CheA to CheY, resulting in phosphorylated CheY. Phospho-CheY then binds proteins in the flagellar motor switch complex (comprised of FliM, FliN, and FliG) to affect flagellar rotational direction (Stock and Surette 1996).

Generally, the basic molecular mechanism of chemotaxis is conserved in bacteria (Wuichet and Zhulin 2010). There is only a single chemotaxis operon in the *E. coli* genome. However, many motile soil bacteria possess two or more chemotaxis operons encoding homologs of the *E. coli* chemotaxis proteins in their genomes (Scharf et al. 2016; Szurmant and Ordal 2004). Furthermore, gene order and composition of operons vary within members of the α -proteobacteria. Chemotaxis operons in some

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*The e-Xtra logo stands for “electronic extra” and indicates that three supplementary movies are published online.

proteobacterial species were reported to regulate chemotaxis response, flagellar synthesis, cell size, cyst formation, biofilms formation, or other cellular functions (Berleman and Bauer 2005; Bible et al. 2008; D'Argenio et al. 2002; Ferrández et al. 2002; Kirby and Zusman 2003; Vlamakis et al. 2004). There are two chemotaxis operons in the genome of *Sinorhizobium meliloti* (which is a synonym of the accepted name *Ensifer meliloti*) and the chemotactic response is controlled by the Che1 pathway (Meier et al. 2007; Sourjik and Schmitt 1996). In *Azospirillum brasilense*, there are four main sets of chemotaxis gene clusters encoded within the genome (Wisniewski-Dyé et al. 2011). Che1 signaling pathway controls swimming speed and has a minor role in chemotaxis (Bible et al. 2012), while Che4 controls changes in swimming direction and competitive colonization on plant root surfaces (Mukherjee et al. 2016).

A. caulinodans ORS571 are motile soil bacteria that have versatile rhizobial lifestyles. Analysis of available genome sequence (Lee et al. 2008) indicates that *A. caulinodans* relies on a single chemotaxis system in the whole genome (Jiang et al. 2016b). The chemotaxis system (*che*) contains five chemotaxis genes, *cheA*, *cheW*, *cheY*, *cheB*, and *cheR*, hereafter called *cheAWY₁BR*, transcribed in the same direction, from a putative σ 54-dependent promoter upstream of *cheA* (Jiang et al. 2016b). While the function of some chemoreceptors has been investigated (Jiang et al. 2016a; Liu et al. 2017a), the role of signaling mediated by the products of the chemotaxis cluster (*che*) in *A. caulinodans* ORS571 has not yet been established. Our results show that the *che* cluster of *A. caulinodans* ORS571 regulates directional motility and other cellular functions in a free-living state or in symbiosis with its host *S. rostrata*. We provide evidence that *che*-mediated regulation of other cellular functions depends on functional flagella-driven motility.

RESULTS

Chemotaxis system of *A. caulinodans* ORS571.

Analysis of the *A. caulinodans* ORS571 genome (Lee et al. 2008) revealed a large DNA region (AZC_0620 to AZC_0666), spanning about 45 kb, that contains open reading frames (ORFs) whose translation products shared similarity with proteins involved in synthesis of flagella and chemotaxis response (Fig. 1A). A preliminary comparative analysis identified a group of five *che* genes (AZC_0661 to AZC_0665), *cheAWY₁BR*, transcribed in the same direction (Fig. 1A). In this gene cluster, translational overlapping is manifest between the *cheA* and *cheW*, between *cheY* and *cheB*, and between *cheB* and *cheR* and, given that chemotaxis proteins are typically organized as operons, it is assumed that the five genes here are also organized as a single transcription unit. A gene (AZC_0660) named *tlpA1*, encoding a transmembrane methyl-accepting chemotaxis protein (MCP) product (Liu et al. 2017a), is located 200 nucleotides upstream of *cheA* (Fig. 1A). Another *mcp* gene (AZC_0666), also encoding a transmembrane MCP product, is located downstream of the *che* cluster (Fig. 1A). The *icpB* gene (AZC_3718) encoding a soluble chemotaxis receptor previously characterized is not linked to the cluster (Jiang et al. 2016a). Two other chemotaxis genes *cheY₂* (AZC_0620) and *cheZ* (AZC_0621), are located in the distal region of the *che* gene cluster and are predicted to be transcribed divergently. A flagella-related gene cluster (AZC_0625 to AZC_0655) containing *fliM* (AZC_0643) is encoded between the two chemotaxis genes (*cheY₂* and *cheZ*) and the *che* gene cluster (Fig. 1A). This favors the hypothesis that the *che* gene cluster encodes a chemotaxis pathway that controls flagella-mediated motility. Furthermore, using transmission electron microscopy, it was found that *A. caulinodans* ORS571 cells possess one to three flagella when grown in liquid medium (Fig. 1B).

A *che* cluster controls chemotaxis response of *A. caulinodans* ORS571.

To determine the role of the *che* cluster in the chemotactic response of *A. caulinodans* ORS571, we constructed two mutant strains (discussed below). The Δ *cheA* mutant is carrying a complete deletion of the *cheA* gene, while a second mutant carries a deletion of the *che* cluster spanning *cheAWY₁BR* and is referred to as Δ *cheA-R*. Both mutants displayed a wild-type flagellation pattern (Fig. 1C and D), while, as expected, a mutant strain carrying a deletion of the *fliM* gene (Δ *fliM*) encoding one of three components of the flagella motor's switch complex was devoid of flagella (Fig. 1E). Chemotaxis behavior of these mutant strains was compared with that of the wild type in soft agar plate assay with carbon sources previously identified as chemoeffectors for *A. caulinodans* ORS571 (Jiang et al. 2016a). In the presence of succinate, lactate, or proline as the sole carbon source and chemoeffector, the Δ *cheA* and Δ *cheA-R* mutant strains expanded from the point of inoculation and formed chemotactic rings that were significantly reduced in size, compared with the wild type, regardless of whether they grew under nitrogen-fixation conditions or with combined nitrogen available (Fig. 2A and B). The chemotaxis defect of the Δ *cheA* mutant strain was rescued by introduction of a plasmid carrying the wild type *cheA* gene into the Δ *cheA* mutant (Δ *cheA*-com) (Fig. 2A and B). Moreover, the chemotaxis pattern displayed by the Δ *cheA* and Δ *cheA-R* mutants in this assay was similar to that of Δ *fliM*, which is a nonmotile mutant strain. These results suggest that the *che* cluster plays an essential role in chemotaxis of *A. caulinodans* ORS571.

To rule out the possibility that the reduced chemotactic ring diameter of the mutants were linked to a defect in bacterial growth, growth rates of each strain was determined in L3 medium (10 mM succinate as carbon source and 10 mM NH₄Cl as nitrogen source). The results show that the growth rate of mutants is similar to that of the wild type (Fig. 3A). Furthermore, since the *cheA* gene is located directly upstream of the *cheWY₁BR* cluster and is transcribed in the same direction (Fig. 1A), we determined whether deletion of *cheA* affected *cheWY₁BR* gene expression by comparing the expression of these genes in the wild type, Δ *cheA*, and Δ *cheA-R* strains and, as expected, found similar expression profiles of *cheWY₁BR* genes in the wild type and Δ *cheA* (Fig. 3B). These data suggest that the diminished diameters observed on swim plate assays are related to defects in chemotaxis response.

To further assess chemotaxis behavior of these mutants, competitive quantitative capillary assays were conducted, using bacterial suspensions containing equal ratios of the wild type and each of these mutants, respectively. Figure 4 shows data obtained with capillary tubes filled with buffer, succinate, or sodium lactate as the attractants. Determination of the number of bacterial cells in capillary tubes filled only with buffer revealed an excess of the wild type as compared with the Δ *cheA*, Δ *cheA-R*, or Δ *fliM* (Fig. 4), suggesting that the swimming motility bias of the mutant cells was impaired. Furthermore, when the capillary tubes were filled with succinate or sodium lactate, the number of the Δ *cheA*, Δ *cheA-R*, or Δ *fliM* mutant cells was also significantly reduced compared with the wild type under similar conditions (Fig. 4). These results confirmed that the Δ *cheA* and Δ *cheA-R* mutants are impaired in cell chemotaxis response and motility behavior.

Involvement of the *cheA* and *che* cluster in the control of flagella-driven motility behavior.

Chemotaxis ultimately controls the probability of change in the swimming direction or swimming motility bias. Chemotaxis mutants are thus expected to display change in the swimming motility bias compared with parent strain. The free-swimming behavior of the wild type and the mutant strains were analyzed by motion tracking. The swimming paths of the

wild-type cells showed their swimming as long runs alternating with sudden direction changes (turns) (Supplementary Movie S1). In contrast, the $\Delta cheA$ (Supplementary Movie S2) and $\Delta cheA-R$ (Supplementary Movie S3) mutants swam with incessant changes in the swimming direction (tumbles). The nonmotile mutant $\Delta fliM$ was used as a control (data not shown). The motility patterns indicate that chemotaxis signaling in *A. caulinodans* ORS571 regulates the probability of change in swimming direction and that the unique Che pathway, through CheA, is the major regulator of this behavior.

The $\Delta cheA$ and $\Delta cheA-R$ mutants affect biofilm formation.

Motility and chemotaxis play important roles in attachment and biofilm formation under static conditions, with the swimming bias being an important trait controlling this behavior (Merritt et al. 2007). The relative biofilm production ability of the wild type and *che* mutant was compared using crystal violet staining (Fig. 5A). Quantitative data further confirmed that the decrease in staining observed with the mutant strains correlated with a decrease in biofilm mass compared with the wild type

Fig. 1. Chemotaxis-related genes encoded within the *Azorhizobium caulinodans* ORS571 genome and flagellation pattern. **A**, Organization of chemotaxis-related genes of *A. caulinodans* ORS571. The arrows indicate the direction of transcription of open reading frames and are drawn relative to scale. **B**, Transmission electron micrographs of *A. caulinodans* ORS571 showing one to three flagella. Cells were negatively stained with phosphotungstic acid. Bar, 2 μ m. **C**, Transmission electron micrographs showing the presence of flagella for the $\Delta cheA-R$ and **D**, $\Delta cheA$ mutant strains and **E**, the absence of flagella for the $\Delta fliM$ mutant.

(Fig. 5B). To further distinguish the possible roles of motility or chemotaxis in biofilm formation, the nonmotility mutant $\Delta fliM$ was also analyzed for its ability to form biofilms and was found to be defective compared with the wild type. These data indicate that the Che signaling pathway modulates biofilm formation, probably through its effect on cell flagella-driven motility.

The $\Delta cheA$ and $\Delta cheA-R$ mutants impaired in biosynthesis of EPS.

EPS are known as key matrix components involved in the process of biofilm formation by bacteria (Ryder et al. 2007; Watnick and Kolter 2000). Thus, EPS production by the $\Delta cheA$ and $\Delta cheA-R$ mutants was compared with that of the wild type. The quantitative results showed that the $\Delta cheA$ and $\Delta cheA-R$ mutants produced less total EPS than the wild type under all tested conditions. Interestingly, the $\Delta fliM$ mutant was also

defective in EPS production (Fig. 6). Taken together, these data indicate that chemotaxis signaling via *che* and flagellar motility modulates EPS production, with this phenotype likely also contributing to the reduced biofilm formation.

The *che* cluster is essential for adsorption and colonization on roots.

Quantitative assays were used to analyze the adsorption and colonization ability of the different strains on the root surfaces to document the role of the *che* cluster in the symbiotic association with the host plant *S. rostrata*. We used a competitive assay to compare the adsorption ability of the mutants and wild type. Surface-sterilized seedlings were co-inoculated with the wild type and mutants at an approximate 1:1 ratio for 4 h. Results indicate that the $\Delta cheA$ and $\Delta cheA-R$ mutants were significantly impaired in their absorption to *S. rostrata* roots

Fig. 2. Chemotaxis behavior of the ORS571 wild type and mutant strains $\Delta cheA-R$ and $\Delta cheA$, the complementation strain $\Delta cheA-com$, and the $\Delta fliM$ mutant toward attractants on soft agar plates. Carbon sources and nitrogen source were included at a final concentration of 10 mM. **A**, Representative L3 plates with succinate as the sole carbon source. **B**, The average diameters of mutants are expressed relative to that of wild type (taken as 100%). Error bars represent standard deviations of mean from three repetitions.

compared with the wild type, whereas the complementation strain ($\Delta cheA$ -com) could restore the phenotype of $\Delta cheA$. In addition, we found that the absorption ability of the $\Delta fliM$ mutant was also severely impaired (Fig. 7A).

We further hypothesized that the lack of adsorption to roots should result in a defective colonization by the $\Delta cheA$ and $\Delta cheA$ -R mutants. First, to establish an overall colonization pattern, a quantitative assay was used to compare the competitive colonization ability of the wild type and the mutants. Three-day-old seedlings were co-inoculated with the wild type and mutants at a 1:1 ratio for 7 days. Colonization efficiency was determined by counting the number of cells re-isolated from the root surfaces at the end of the incubation period. As expected, the number of cells of the $\Delta cheA$ and $\Delta cheA$ -R mutants recovered from the root surface was significantly reduced compared with wild-type cells. However, the wild-type phenotype could be restored in the complementation strain $\Delta cheA$ -

com. Similarly, the $\Delta fliM$ mutant deficient in flagella was shown to be less competitive in colonization of the host (Fig. 7B). Given that both the $\Delta cheA$ and $\Delta cheA$ -R mutants as well as the nonflagellated $\Delta fliM$ mutant are severely impaired in adsorption and surface colonization of *S. rostrata* roots, this suggests that the Che signaling pathway contributes to root symbiosis through regulating flagella-based motility.

The Che pathway modulates competitive nodule formation.

Surface colonization is essential for effective symbiotic nodulation of *Sesbania* spp. (Mitra et al. 2016). To further test whether the chemotaxis mutants have any symbiotic nodulation defects, the nodule formation ability was used to compare between the wild type and mutants. When inoculated alone on stems or roots of *S. rostrata*, the $\Delta cheA$ and $\Delta cheA$ -R mutants formed normal stem (Fig. 8A) or root nodules (Fig. 8B), with

Fig. 3. Growth of ORS571 and mutant strains and gene expression. **A**, Growth curves of the wild type (WT), $\Delta cheA$ -R, $\Delta cheA$, and $\Delta fliM$ in L3 medium. **B**, Expression of *cheWY1BR* genes in the wild-type (ORS571) and chemotaxis mutants. The *cheWY1BR* gene expression was detected by polymerase chain reaction using appropriate primers. The *16S rRNA* gene was amplified as a positive gene control and the *cheA* gene was amplified as a negative gene control. + indicates samples incubated with reverse transcription and - indicates samples with no reverse transcription.

Fig. 4. Competitive quantitative capillary chemotaxis assays of the ORS571 wild type, $\Delta cheA$, $\Delta cheA$ -R, and $\Delta fliM$ mutants. The capillary tubes filled with chemotaxis buffer (left), 10 mM succinate (middle), or sodium lactate (right) as the carbon source were then inserted into the 96-well plate containing the bacterial suspension (mutants and wild type 1:1 ratio). Each datapoint represents the mean of three independent experiments and the error bars represent the standard error of the mean.

no obvious difference in the morphology, compared with the wild type. Further, the competitive nodulation ability on stems and roots was used to compare between the wild type and $\Delta cheA$ or $\Delta cheA-R$ or $\Delta flhM$ mutants. All these mutants were severely defective in the ability to compete for nodulation of root and stem compared with the wild type (Fig. 8C). Together, these results demonstrate that Che pathway-mediated flagella motility is important for promoting competitive nodule formation.

DISCUSSION

Many motile soil bacteria possess multiple chemotaxis operons playing pleiotropic roles in regulating flagella-mediated motility and other functions (Scharf et al. 2016). *A. caulinodans* is a motile, nitrogen-fixing soil bacterium with a preference for organic acids as carbon source (Dreyfus et al. 1988). There is a single chemotaxis gene cluster (*cheAWY₁BR*) (Fig. 1A) predicted from the genome sequence, but the DNA region containing this cluster contains other genes involved also in the chemotactic response, such as *tlpA1* (AZC_0660) (Liu et al.

2017a) and *cheZ* (AZC_0621) (Liu et al. 2017b). Here, we confirmed that the Che pathway (*cheAWY₁BR*) directly controls the cellular chemotaxis response and flagella-mediated motility behavior. A mutant lacking partial *cheA* (Liu et al. 2017a) does not yield a null chemotaxis phenotype in soft agar plates (data not shown), but there is no obvious difference in capillary tube chemotaxis assays and flagella motility behavior (Liu et al. 2017a) compared with $\Delta cheA-R$ mutants. This is why, in the present work, the role of CheA was re-evaluated using a new construction carrying a complete deletion of the *cheA* gene ($\Delta cheA$). Indeed, the chemotaxis phenotype and motility behavior of the newly constructed $\Delta cheA$ mutant is similar to that of the mutant strain carrying the complete deletion of the *che* cluster $\Delta cheA-R$ (Fig. 2). This indicates that chemotaxis signaling in *A. caulinodans* ORS571 controls the chemotaxis response and flagella-driven motility and that the unique *che* cluster, through the unique CheA, is the major regulator of these behaviors.

Che operons appear to regulate different cell functions in different bacteria (Berleman and Bauer 2005; D'Argenio et al.

Fig. 5. Biofilms generated by ORS571 wild type, $\Delta cheA$, $\Delta cheA-R$, and $\Delta flhM$ mutants. **A**, Bacterial strains were grown in glass tubes containing L3 (10 mM succinate as carbon source) medium for 3 to 5 days without shaking. Biofilms were visualized by staining with crystal violet. **B**, Quantification of crystal violet staining was determined at the optical density at 570 nm wavelength. The error bars represent standard errors from the mean of five repetitions. Asterisks indicate statistically significant differences ($P \leq 0.05$) between the wild type and the mutants.

2002; Ferrández et al. 2002; Kirby and Zusman 2003; Vlamakis et al. 2004). In the *Azospirillum* genus, the Che1 pathway of *Azospirillum brasilense* also encodes a conserved set of CheA-WYBR homologs controlling swimming speed during chemotaxis and regulating the cell size (Bible et al. 2008; Gullett et al. 2017). In addition, several *che* operon mutants of *Azospirillum brasilense* had an altered production of total EPS that affected colony morphology as well as clumping and flocculation behaviors (Bible et al. 2008). In the present work, the flocculation behavior showed no significant difference between wild type and mutants (data not shown). However, our data showed that defects in chemotaxis caused by mutation within *che* genes affected chemotaxis response as well as the total amount of EPS produced.

Interestingly, decreased EPS biosynthesis in the $\Delta fliM$ mutant is similar to that observed in the *che* mutants. Given that a

nonflagellated mutant had a similar phenotype to nonchemotactic mutants, this suggests that the $\Delta cheA$ - and $\Delta cheA-R$ -reduced EPS production is likely indirectly related to chemotaxis behavior and, rather, is associated with flagella-driven motility. Several other genes involved in the chemotactic response in *A. caulinodans* were also shown to be involved in EPS production. In particular, a mutation of the *icpB* gene, encoding a soluble chemoreceptor and mutation of the *cheZ*-like gene, led to increased EPS production (Jiang et al. 2016a, Liu et al. 2017b) suggesting their involvement in a negative regulation of EPS production, while the Che pathway and FliM would be involved in a positive regulation of EPS.

The decreased EPS production of $\Delta cheA$ and $\Delta cheA-R$ and nonflagellated ($\Delta fliM$) mutant is correlated with decreased biofilm formation (Fig. 5), as also reported for a mutant of the

Fig. 6. Quantitative analysis of extracellular polysaccharide (EPS) production of the ORS571 wild type, $\Delta cheA$, $\Delta cheA-R$, and $\Delta fliM$ on plates with different carbon sources. Error bars indicate standard deviations from the mean of three independent experiments.

Fig. 7. Adsorption and colonization ability of the *Azorhizobium caulinodans* ORS571 wild type and chemotaxis mutant strains on *Sesbania rostrata* root surface. **A**, Competitive adsorption on *S. rostrata* roots with the wild type and either $\Delta cheA$, $\Delta cheA-R$, $\Delta fliM$, or complementation strain $\Delta cheA-com$ in an approximate 1:1 ratio. **B**, Competitive colonization levels of *S. rostrata* roots with the wild type and either $\Delta cheA$, $\Delta cheA-R$, $\Delta fliM$, or complementation strain $\Delta cheA-com$ in an approximate 1:1 ratio. The adsorption and colonization ratios were analyzed by polymerase chain reaction. Error bars represent standard errors of the mean calculated from three independent experiments.

transmembrane chemoreceptor *tlpA1* (Liu et al. 2017a), while, in contrast, the *cheZ*-like mutant strain displayed increased ability of biofilm formation. This suggests a complex regulatory network that needs to be further investigated. In *E. coli*, biofilm formation in $\Delta cheA$ -Z (nonchemotactic) was similar to that of the wild type, and only the nonflagellated or paralyzed flagellated mutants were defective in formation of biofilms. This finding indicates bacterial motility but not chemotaxis is essential for biofilm formation in *E. coli* (Pratt and Kolter 1998), but the chemotaxis and its effect on the swimming-motility pattern appear to be important in biofilm formation by several soil bacteria, such as *Azorhizobium caulinodans* and *Azospirillum brasilense* but an exact mechanism is yet to be identified.

Previous research indicates that chemotaxis and motility can confer a significant advantage of bacterial competitiveness in the rhizosphere (Brencic and Winans 2005), colonization on plant roots (Bernabéu-Roda et al. 2015; Dekkers et al. 1998; de Weger et al. 1997; Miller et al. 2007; Simons et al. 1996; Van de Broek and Vanderleyden 1995; Van de Broek et al. 1998), and the efficiency of nodule initiation (Caetano-Anollés et al. 1988). In *Sinorhizobium meliloti*, several nonmotile or nonchemotactic mutants (*che*⁻, *mof*⁻, and *fla*⁻) were all less effective than the parent as competitors in root adsorption and nodulation, and motility and chemotaxis appear to be quantitatively important in root adsorption and nodule initiation (Caetano-Anollés et al. 1988). In *Rhizobium leguminosarum*, only the

Fig. 8. Nodulation tests between the *Azorhizobium caulinodans* ORS571 wild type and mutants. **A**, Stem nodules were induced by the wild type (WT), $\Delta cheA$, and $\Delta cheA$ -R. Leghemoglobin of stem nodules shows characteristic orange-brown color. **B**, Root nodules induced by WT, $\Delta cheA$, and $\Delta cheA$ -R mutants. **C**, Analysis of numbers of WT, $\Delta cheA$, $\Delta cheA$ -R, $\Delta fliM$, and complementation strain ($\Delta cheA$ -com) nodules in a competitive nodulation assay on stems and roots. Error bars represent standard errors of the mean calculated from three independent experiments.

che1 cluster controls swimming motility bias and chemotaxis as well as competitive nodule formation, while the *che2* cluster that has a minor effect on chemotaxis is dispensable for competitive nodulation. In *Azospirillum brasilense*, the Che1 chemotaxis pathway has a minor role in chemotaxis and root-surface colonization. However, the Che4 pathway regulates the swimming pattern of motile *Azospirillum brasilense* cells and wheat root-surface colonization. The Che4 pathway is orthologous to the major pathway controlling all chemotaxis responses in *Sinorhizobium meliloti* (Armitage and Schmitt 1997) and *Rhizobium leguminosarum* (Caetano-Anollés et al. 1988), in which this chemotaxis system is also essential for plant root colonization. In this study, although the $\Delta cheA$ and $\Delta cheA$ -R mutants are not impaired in their absorption and nodulation ability after inoculation alone, all these mutants appeared to be severely impaired in their competitive colonization and nodulation behavior, suggesting that these genes positively control efficient nodulation, as also reported for IcpB and TlpA1. In contrast, CheZ was found to have a negative role (Liu et al. 2017b). In addition, the nonmotility ($\Delta fliM$) mutants were also severely defective in competitive nodule formation on roots and stems of the host plant. These results suggest that the chemotaxis signal transduction pathway controls flagella-driven motility, which regulates competitiveness in the rhizosphere.

In establishment of a successful symbiosis between rhizobia and legumes, in addition to chemotaxis and motility, bacterial surface lipopolysaccharides and EPS are essential for colonization and nodule development (Gao et al. 2001; Mathis et al. 2005; Mitra et al. 2016). The results reported here support the conclusion that the Che signaling pathway in *A. caulinodans* not only provide a competitive advantage in root colonization by regulating chemotaxis and mediating flagella-driven but also indirectly affected competitive colonization through affection biofilm formation or EPS biosynthesis.

MATERIALS AND METHODS

Strains and growth condition.

All strains and plasmids used in this study are listed in Table 1. *A. caulinodans* ORS571 wild-type strain and its derivatives were grown in L3 or tryptone yeast (TY) liquid medium at 37°C with shaking (180 rpm). The L3 medium is supplemented or not with 10 mM NH₄Cl. L3 and TY medium containing, per milliliter, 100 µg of ampicillin and 25 µg of nalidixic acid were prepared as described previously (Jiang et al. 2016a). *E. coli* was grown in LB medium or agar plates at 37°C.

Construction of mutants and complementation strain.

To construct a marker-less *che* gene cluster deletion mutant (deletion of *cheA*, *cheW*, *cheY*, *cheB*, and *cheR* genes), a 825-bp

upstream fragment (UF) was amplified by polymerase chain reaction (PCR) from the genomic DNA of *A. caulinodans* ORS571, using primers CheUF and CheUR (Table 2), and a 801-bp downstream fragment (DF) was amplified by PCR using primers CheDF and CheDR (Table 2). The PCR product corresponding to the upstream DNA fragment was digested with *EcoRI*-*NdeI* and was then inserted into the pCM351 plasmid (Marx and Lidstrom 2002) digested with *EcoRI*-*NdeI*. The resulting plasmid was designated as pCM351::UF. The PCR product corresponding to the DF was digested with *SacII*-*SacI* and was cloned into pCM351::UF. The final plasmid, pCM351::UF::DF, was transformed into *E. coli* DH5 α and the DNA sequence was verified by sequencing, prior to mating. The recombinant plasmid was transferred into *A. caulinodans* ORS571 by triparental conjugation with the helper plasmid pRK2013 (Figurski and Helinski 1979). Recombinants from double homologous recombination were obtained and were screened on TY plates by selecting for gentamicin resistance and tetracycline sensitivity (Marx and Lidstrom, 2002). The potential mutant was generated by in-frame deletion of an internal 5,756-bp fragment and insertion of a gentamicin-resistance gene. The gentamicin gene was then deleted by introduction of the Cre expression plasmid pCM157 (Marx and Lidstrom 2002). The correct mutation was verified by PCR with primer pair CheAF and CheAR (Table 2), and a confirmed mutation was named $\Delta cheA$ -R.

To construct a complete deletion mutant of the *cheA* gene, a 501-bp UF was amplified using primer pair CheAUF and CheAUR (Table 2), and a 576-bp DF was amplified by PCR using primers CheADF and CheADR (Table 2). The PCR product of the upstream DNA fragment was digested with *NsiI*-*NdeI* and was then inserted into the pCM351 plasmid digested with *NsiI*-*NdeI*. The resulting plasmid was termed pCM351::UF. The PCR product corresponding to the DF was digested with *ApaI*-*AgeI* and was cloned into pCM351::UF. Plasmid pCM351::UF::DF was transformed into *E. coli* DH5 α and the DNA sequence was verified by sequencing. The recombinant plasmid was transferred into *A. caulinodans* ORS571 by triparental conjugation. The potential mutant was generated by in-frame deletion of an internal 2,793-bp fragment and insertion of a gentamicin-resistance gene. Then, the gentamicin gene was deleted by introduction of plasmid pCM157 (Marx and Lidstrom 2002). The introduction of the correct mutation was verified by PCR with primer pair CheAF and CheAR (Table 2), and a confirmed mutation was named $\Delta cheA$.

For complementation of $\Delta cheA$, the *cheA* ORF with 601 bp immediately upstream from the noncoding sequence was amplified by PCR, using primer pair CheACF and CheACR (Table 2). The amplified 3,394-bp fragment was inserted into *SpeI* and *XbaI* sites of the broad host-range cloning vector pBBR1MCS-2

Table 1. Strains and plasmids used in this study

Strain or plasmid	Relevant properties ^a	Source or reference
<i>Azorhizobium caulinodans</i> ORS571	Wild-type strain, Amp ^r , Nal ^r	Dreyfus et al. 1988
$\Delta cheA$ -R	ORS571 derivative, <i>che</i> deletion mutant, Amp ^r , Nal ^r	This study
$\Delta cheA$	ORS571 derivative, <i>cheA</i> complete deletion mutant, Amp ^r , Nal ^r	This study
$\Delta fliM$	ORS571 derivative, <i>fliM</i> deletion mutant, Amp ^r , Nal ^r	This study
WT-pBBR	Wild type strain carrying pBBR1MCS-2, Amp ^r , Nal ^r , Kan ^r	This study
$\Delta cheA$ -com	ORS571 derivative, <i>cheA</i> deletion mutant carrying pBBR1MCS-2- <i>cheA</i> , Amp ^r , Nal ^r , Kan ^r	This study
<i>Escherichia coli</i> DH5 α	F ⁻ SupE44 $\Delta lacU169$ $\phi 80 lacZ\Delta M15$ hsdR17 <i>recA1 endA1 gyrA96 thi-1 relA1</i>	Transgen
pCM351	Mobilizable allelic exchange vector, Amp ^r , Gen ^r	Marx and Lidstrom 2002
pCM157	IncP plasmid that expresses Cre recombinase, Tet ^r	Marx and Lidstrom 2002
pBBR1MCS-2	Broad host-range plasmid, Kan ^r	Kovach et al. 1995
pBBR- <i>cheA</i>	pBBR1MCS-2 carrying the native promoter of the <i>cheA</i> and <i>cheA</i> gene, Kan ^r	This study
pRK2013	Helper plasmid, ColE1 replicon, Tra ⁺ , Kan ^r	Figurski and Helinski 1979

^a Amp^r, Nal^r, Gen^r, Kan^r, and Tet^r indicate ampicillin, nalidixic acid, gentamicin, kanamycin, and tetracycline resistance, respectively.

(Kovach et al. 1995). The ligation mixture was transformed into *E. coli* DH5 α . DNA sequences from individual clones were sequenced and one verified clone was designated as pBBR1MCS-2-*cheA*-com. This recombinant plasmid, named pBBR-*cheA*, was then introduced into the Δ *cheA* mutant via triparental mating and the transformants were recovered by selection for kanamycin resistance. One of the resulting strains was designated as Δ *cheA*-com.

To construct a *fliM* gene-deletion mutant, a 695-bp UF was amplified by PCR from the genomic DNA of *A. caulinodans* ORS571, using primer pair FliMUF and FliMUR (Table 2), and a 579-bp DF was amplified by PCR, using primers FliMDF and FliMDR (Table 2). The PCR product corresponding to the upstream DNA fragment was digested with *KpnI*-*NdeI* and was then inserted into the pCM351 plasmid digested with *KpnI*-*NdeI*. The resulting plasmid was termed as pCM351::UF. The PCR product corresponding to the DF was digested with *AgeI*-*SacI* and was cloned into pCM351::UF. A final plasmid, pCM351::UF::DF, was transformed into *E. coli* DH5 α and the DNA sequence was verified by sequencing, prior to mating. The recombinant plasmid was transferred into *A. caulinodans* ORS571 by triparental conjugation. The potential mutant was generated by in-frame deletion of an internal 930-bp fragment and insertion of a gentamicin-resistance gene. The gentamicin gene was then deleted (Marx and Lidstrom 2002). The mutation was verified by PCR with primer pair FliMF and FliMR (Table 2) and a confirmed mutation was named Δ *fliM*.

Electron microscopy.

Strains were grown overnight with shaking at 37°C in TY to mid-logarithmic phase. Cultures were inspected to ensure motility, and cells were taken directly from the cultures and adsorbed to Formvar-coated copper grids for 5 min. Excess culture was blotted with filter paper and the grid was placed face down on a drop of 1% phosphotungstic acid for 1 min. Excess

stain was blotted with filter paper, and the grid was dried. The images were obtained using a JEM-1400 electron microscope.

Behavioral assays.

Soft agar plates were used to detect chemotactic responses of the wild type and mutants to different carbon sources. L3 plates (0.35% agar) supplemented with 10 mM carbon source (succinate, sodium lactate, or proline) as attractant and containing or not 10 mM NH₄Cl as nitrogen source. Cells were grown up to mid-exponential phase in TY medium and were harvested by centrifugation. The cultures were washed twice and were then resuspended in the chemotaxis buffer (10 mM K₂HPO₄, 10 mM KH₂PO₄, 0.1 mM EDTA, pH 7.0) to give an optical density at 600 nm (OD₆₀₀) of 1.0. Aliquots of a 5- μ l suspension were used to inoculate on L3 plates. The plates were incubated at 37° for 48 to 72 h and, then, swimming diameters were recorded.

Quantitative capillary chemotaxis assays were performed as previously described by Reyes-Darias et al. (2016) with some minor modifications. The wild type and mutants were grown to early logarithmic phase (OD₆₀₀ of 0.3 to 0.4) in TY medium. The cultures were washed and were resuspended with chemotaxis buffer to OD₆₀₀ = 0.05. Then, 300- μ l aliquots of cell suspensions (1:1 ratio of wild-type to mutant) were added into each well of a 96-well plate. The open end of capillary tubes (containing 10 mM succinate or sodium lactate as attractant) was placed into the wells. Capillary tubes filled with chemotaxis buffer were used as controls. Subsequently, the sealed end was broken and the contents were emptied into 1 ml of sterile water, after incubation for 1 h. Serial 10-fold dilutions were placed onto TY plates. Colonies (n = 480) randomly selected on TY plates were analyzed by PCR.

To observe the swimming behavior of cells, cultures were grown in TY medium to mid-exponential phase and the swimming behavior was observed and recorded using Olympus DP73 digital microscope camera. Videos were analyzed to

Table 2. Primers used in this study^a

Primer	Sequence (5'-3')	Purpose
CheUF- <i>EcoRI</i>	GGAATTCCGAGATCGGTACCCAGGTG	<i>ΔcheA-R</i> mutant construction
CheUR- <i>NdeI</i>	GGAATTCATATGCTGTCACTCCAGCGGGCTG	<i>ΔcheA-R</i> mutant construction
CheDF- <i>SacII</i>	TCCC CGCGGGGACATAAACCCAAGGATAG	<i>ΔcheA-R</i> mutant construction
CheDR- <i>SacI</i>	CGAGCTCCGTCACGATGCTGCGGAAG	<i>ΔcheA-R</i> mutant construction
CheAF	CAGGAAGACTCCGAATACAAGGT	Validation of <i>cheA</i> or <i>che</i>
CheAR	GATCATCTCGATGTTTCGAGCG	Validation of <i>cheA</i> or <i>che</i>
CheAUF- <i>NsiI</i>	ATGCATGGACACCATCCGCAAGATTGAC	<i>ΔcheA</i> construction
CheAUR- <i>NdeI</i>	CATATGCTCGCGCAGCAGATCATCCAT	<i>ΔcheA</i> construction
CheADF- <i>ApaI</i>	GGGCCCGCCCATGGAGAGGCGGCATGA	<i>ΔcheA</i> construction
CheADR- <i>AgeI</i>	ACCGGTCACACGCGCTGAATCATCCAC	<i>ΔcheA</i> construction
CheACF- <i>SpeI</i>	ACTAGTTGGCGAGCGAAGTGAAGTC	<i>ΔcheA</i> -com construction
CheACR- <i>XbaI</i>	TCTAGAGTCGAACCTGGCGATGTAGTC	<i>ΔcheA</i> -com construction
FliMUF- <i>KpnI</i>	GGTACCCGACCGAGCTTCATGAGAT	Δ <i>fliM</i> construction
FliMUR- <i>NdeI</i>	CATATGGCGGGTCCGAAGAGCAGATC	Δ <i>fliM</i> construction
FliMDF- <i>AgeI</i>	ACCGGTCATCGAGCAGCATGTCT	Δ <i>fliM</i> construction
FliMDR- <i>SacI</i>	GAGCTCGACGCAGTATATCTGCGAC	Δ <i>fliM</i> construction
FliMF	ACATAAGCGCCTTCGGCCT	Validation of <i>fliM</i>
FliMR	GTCATCTTCGACCGCCTGG	Validation of <i>fliM</i>
CheAQF	GTCTCGTGGTGGACGAGATT	RT-PCR for <i>cheA</i>
CheAQR	TGAGGTTGCGGTAGAAGAGG	RT-PCR for <i>cheA</i>
CheWQF	GTCTTCGTACCGGACCACAT	RT-PCR for <i>cheW</i>
CheWQR	ACCTCGTCGATCATGATCC	RT-PCR for <i>cheW</i>
CheY ₁ QF	CCGACTGGAACATGGAGCCGAT	RT-PCR for <i>cheY₁</i>
CheY ₁ QR	TCAGGCCTCGAACACGGTGTG	RT-PCR for <i>cheY₁</i>
CheBQF	ACATGGTGTGGAGAAGTCC	RT-PCR for <i>cheB</i>
CheBQR	AACGACGCTTGTTCTTCGT	RT-PCR for <i>cheB</i>
CheRQF	CGCTGGCGATGACGCTGAAG	RT-PCR for <i>cheR</i>
CheRQR	AGGCCACGAACGGGTTGAG	RT-PCR for <i>cheR</i>
16SQF	ACGGATTTCTCCAGCAATG	RT-PCR for 16S rRNA
16SQR	ACCGGCAGTCCCTTTAGAGT	RT-PCR for 16S rRNA

^a Engineered restriction sites are underlined; RT-PCR = reverse transcription-polymerase chain reaction.

observe the cell paths for 2 to 3 s. For each strain, at least five independent experiments were observed.

Reverse transcription PCR.

Total RNA was isolated from cultured free-living cells of wild type and mutants. cDNA was generated using the TransScript all-in-one first-strand cDNA synthesis supermix for quantitative PCR (one-step genomic DNA removal) kit (Transgen) and was diluted fivefold as template for subsequent PCR amplification. PCR was performed with gene-specific primer pairs (Table 2). The PCR program included an initial denaturation step at 95°C for 3 min, followed by 30 cycles of 95°C for 30 s, 62°C for 30 s, and 72°C for 30 s.

Biofilm assays.

To form biofilm on abiotic surfaces (glass tubes), cell cultures (OD₆₀₀ of 2.5) were resuspended in L3 medium. Cultures (150 µl) were inoculated into 1.5-ml glass tubes at 37°C for 3 to 5 days. For crystal violet staining, cultures were removed and were washed with double-distilled (dd)H₂O three to five times. Then, 0.5% crystal violet was added into each tube. After incubation for 20 min, the crystal violet was removed and was washed with ddH₂O five times, the water was removed completely in the final wash, and 1.5 ml of 30% acetic acid was added to dissolve the “crystal violet ring” off and the OD₅₇₀ was determined.

EPS production assay.

For the examination of EPS production, overnight cultures were adjusted to an OD₆₀₀ of 1.0, and 15 µl of bacterial suspension was inoculated onto solid L3 plates (0.8% agar) and was then incubated at 37°C for 1 to 3 days. L3 plates contained 10 mM of different carbon sources and were supplemented or not with 10 mM NH₄Cl as the nitrogen source. Quantification of EPS production was performed based on the method of Nakajima et al. (2012), with some minor modifications. Bacteria on the L3 plates were collected and were resuspended in λ-buffer (10 mM Tris-HCl, pH 7.0, 10 mM MgSO₄). Supernatant containing the EPS soluble fraction were first treated with 1 ml of concentrated sulfuric acid containing 0.2% anthrone, mixed, and incubated for 7 min at 100°C, and then, were quickly chilled on ice. The OD₆₂₀ of the mixture was measured on a Nanodrop 2000C. D-glucose was used for the standard curve.

Adsorption and colonization assay of roots.

The number of root-adsorbed rhizobia was determined as described by Zamudio and Bastarrachea (1994), with some minor modifications. *S. rostrata* seeds were germinated and grown under sterile conditions for 3 days. Cultures of each strain (OD₆₀₀ nm of 0.2) were used to inoculate seedlings for 4 h at 28°C, with slow shaking, at 50 rpm. The slow shaking was included to reduce the likelihood that motility differences account for differences in reaching the root surfaces. Then, the seedlings were washed by gentle suspension with sterile water shaking in the rotary bath at 100 rpm for 1 min. Five consecutive washes were performed to remove the excess bacteria. The terminal 2 cm of the inoculated roots of five plants were dispersed in sterile water by a homogenizer and the dilution was spread on TY agar plates. The adsorption pattern of the root surfaces was examined by counting the colony-forming units on TY agar plates. In the competitive adsorption experiment, surface-sterilized seedlings were inoculated with mixtures of wild type and mutants at approximate 1:1 for 4 h.

Colonization assays were carried out as described by Greer-Phillips et al. (2004), with a few modifications. Wild type and mutants were mixed 1:1 and were added to glass bottles

containing 200 ml of molten agar (0.35% agar). Ten surface-sterilized seedlings were embedded into each bottle and were incubated at 30°C. After 7 days, the roots were washed and were homogenized by homogenizer. Serial dilutions of the supernatant were spread on TY agar plates to count colony-forming units. Colonies were further assigned as detected by PCR. A non-inoculated control flask was included for all experiments.

Nodulation assays.

Nodulation and competition assays were carried out as described by Yost et al. (1998), with the following modifications. For nodulation assays on roots, surface-sterilized seedlings were co-inoculated with each strain in an approximate OD₆₀₀ of 0.5. For stem nodulation assays, stems root primordial were inoculated with the bacterial suspension by painting using sterile cotton wool. For competitive nodulation assays on roots and stems, surface-sterilized seedlings and plant stems were co-inoculated with wild type and mutants in an approximate 1:1 ratio. All plants were grown at 27°C in the greenhouse, with a daylight illumination period of 12 h. Stem nodules were harvested 20 to 30 days postinoculation. Bacteria were re-isolated from surface-sterilized nodules and colonies were identified by PCR. Controls included uninoculated plants.

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