CheY1 and CheY2 of *Azorhizobium caulinodans* ORS571 Regulate Chemotaxis and Competitive Colonization with the Host Plant

Wei Liu,^{a,b} Xue Bai,^{a,c} Yan Li,^{a,b} Jun Min,^{a,b} Yachao Kong,^{a,b} Xiaoke Hu^{a,b}

^aKey Laboratory of Coastal Biology and Bioresource Utilization, Yantai Institute of Coastal Zone Research, Chinese Academy of Sciences, Yantai, China

^bLaboratory for Marine Biology and Biotechnology, Qingdao National Laboratory for Marine Science and Technology, Qingdao, China

^cCollege of Life Sciences, Yantai University, Yantai, China

ABSTRACT The genome of *Azorhizobium caulinodans* ORS571 encodes two chemotaxis response regulators: CheY1 and CheY2. *cheY1* is located in a chemotaxis cluster (*cheAWY1BR*), while *cheY2* is located 37 kb upstream of the *cheAWY1BR* cluster. To determine the contributions of CheY1 and CheY2, we compared the wild type (WT) and mutants in the free-living state and in symbiosis with the host *Sesbania rostrata*. Swim plate tests and capillary assays revealed that both CheY1 and CheY2 play roles in chemotaxis, with CheY2 having a more prominent role than CheY1. In an analysis of the swimming paths of free-swimming cells, the $\Delta cheY1$ mutant exhibited decreased frequency of direction reversal, whereas the $\Delta cheY2$ mutant appeared to change direction much more frequently than the WT. Exopolysaccharide (EPS) production in the $\Delta cheY1$ and $\Delta cheY2$ mutants was lower than that in the WT, but the $\Delta cheY2$ mutant had more obvious EPS defects that were similar to those of the $\Delta cheY1$ $\Delta cheY2$ and $\Delta eps1$ mutants. During symbiosis, the levels of competitiveness for root colonization and nodule occupation of $\Delta cheY1$ and $\Delta cheY2$ mutants were impaired compared to those of the WT. Moreover, the competitive colonization ability of the $\Delta cheY2$ mutant was severely impaired compared to that of the $\Delta cheY1$ mutant. Taken together, the $\Delta cheY2$ phenotypes are more severe than the $\Delta cheY1$ phenotype in free-living and symbiotic states, and that of the double mutant resembles the $\Delta cheY2$ single-mutant phenotype. These defects of $\Delta cheY1$ and $\Delta cheY2$ mutants were restored to the WT phenotype by complementation. These results suggest that there are different regulatory mechanisms of CheY1 and CheY2 and that CheY2 is a key chemotaxis regulator under free-living and symbiosis conditions.

IMPORTANCE *Azorhizobium caulinodans* ORS571 is a motile soil bacterium that has the dual capacity to fix nitrogen both under free-living conditions and in symbiosis with *Sesbania rostrata*, forming nitrogen-fixing root and stem nodules. Bacterial chemotaxis to chemoattractants derived from host roots promotes infection and subsequent nodule formation by directing rhizobia to appropriate sites of infection. In this work, we identified and demonstrated that CheY2, a chemotactic response regulator encoded by a gene outside the chemotaxis cluster, is required for chemotaxis and multiple other cell phenotypes. CheY1, encoded by a gene in the chemotaxis cluster, also plays a role in chemotaxis. Two response regulators mediate bacterial chemotaxis and motility in different ways. This work extends the understanding of the role of multiple response regulators in Gram-negative bacteria.

KEYWORDS *Azorhizobium caulinodans*, response regulator CheY, chemotaxis, symbiosis

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Address correspondence to Xiaoke Hu, xkhu@yic.ac.cn.

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Bacterial chemotaxis is a response to environmental signals in which cells adjust the direction of flagellar rotation to swim toward chemical attractors and away from repellents (1). Chemotaxis and motility provide a competitive advantage to motile soil bacteria in the colonization of plant root surfaces (2). Chemotactic signal transduction from transmembrane chemoreceptors to flagellar motors is mediated by the CheA/CheY two-component regulatory system (3). Transmembrane chemoreceptors sense signals in the periplasm and transmit the activating signal to the receptor-related cytoplasmic proteins CheW and CheA. The chemoreceptor signals activate the histidine kinase activity and phosphorylation of CheA, using ATP as the phosphodonor (4). Then, CheA receives signals from receptors and transfers the phosphoryl group to the response regulator CheY protein (5).

Response regulator CheYs are the switch elements of two-component systems. The fraction of the response regulator population that is phosphorylated at any given time is determined by the balance between the rates of phosphoryl group addition (CheY-phosphate [CheY-P]) and removal (CheY) (6). CheY-P has an enhanced affinity for the FliM component of the motor switch complex, causing the flagella to switch from counterclockwise to clockwise rotation (6). Counterclockwise rotation causes cells to swim in a straight line, whereas clockwise rotation causes cells to tumble and randomly change their direction of motility (7). Thus, mutants that cannot make CheY-P (*cheW*, *cheA*, or *cheY* mutants) typically exhibit smooth swimming, whereas those that have elevated levels of CheY-P (*cheZ* mutant) tumble (8). The chemotaxis response regulator CheYs contain a conserved receiver domain active site consisting of conserved amino acids that catalyze signal phosphorylation and dephosphorylation reactions (9). In *Escherichia coli*, six highly conserved amino acids are Asp12, Asp13, Asp57, Gly65, Thr87, and Lys109. The receiver domain active sites Asp12, Asp13, and Asp57 bind divalent cations that are required for phosphorylation and dephosphorylation reactions (10, 11). Thr87 (12) and Lys109 (13) play direct roles in the catalysis of phosphotransfer (9). In addition, CheY crystal structures show that the amino acid distribution at position T (Thr) 87+1 influences the kinetics of response regulator phosphorylation and dephosphorylation (14).

Genomic and bioinformatic analyses of more than 450 bacteria indicate that more than 50% of the chemotaxis gene homologs have multiple copies (15) and that these genes not only are involved in flagellum-mediated chemotaxis but also can regulate type IV pilus-based motility (16), flagellar morphogenesis (17), and polysaccharide biosynthesis (18). Unlike the *E. coli* two-component chemosensory pathway, which relies on a single copy of the response regulator CheY, multiple homologs of CheY are not unusual in many bacteria. Recent studies have demonstrated that multiple copies of CheY play specific roles in chemotactic signal transduction mechanisms. For example, *Vibrio cholerae* contains four copies of the chemotaxis response regulator, and only CheY3 directly controls flagellar rotation (19). In *Borrelia burgdorferi*, only CheY3 can change motor action, while the other two cannot bind to the motor or act as signal regulators for phosphate reactions (20). Similarly, in *Rhodobacter sphaeroides*, only CheY6 and either CheY3 or CheY4 are important for controlling flagellar motor rotation (21). In addition, multiple CheYs have been found in nitrogen-fixing soil bacteria, such as *Rhizobium leguminosarum*, *Sinorhizobium meliloti*, and *Azospirillum brasilense*. *R. leguminosarum* contains two chemotaxis gene clusters that encode three response regulators (22). *S. meliloti* involves two response regulators, namely, CheY1 and CheY2, both of which are phosphorylated by CheA. CheY2-P is a regulator of motor function that causes a decrease in the rotary speed of the unidirectional (clockwise) flagellar motor (23). CheY2-P retrotransfers the phosphoryl group to CheA, which in turn phosphorylates CheY1 (24). CheY1 in conjunction with unphosphorylated CheA acts as a sink for phosphoryl groups from CheY2-P and therefore emulates the role of the phosphatase CheZ (25). *A. brasilense* Sp7 possesses seven response regulators. Che4 regulates swimming reversal, chemotaxis, and wheat root surface colonization. However, CheY1 regulates only swimming speed (26). In recent studies, the *cheY7* mutant

FIG 1 Organization of chemotaxis response regulator genes *cheY1* and *cheY2* in genome of *A. caulinodans* ORS571. The arrows indicate the direction of transcription of open reading frames and are drawn relative to scale.

strain showed transient pauses, and the frequency of the transient pauses increased dramatically in the absence of CheY4 (27).

Azorhizobium caulinodans ORS571 is a motile soil bacterium that can induce the formation of effective nitrogen-fixing nodules on the roots and stems of the host plant *Sesbania rostrata* (28). *A. caulinodans* ORS571 cells possess one to three flagella when grown in liquid medium (18). The genome of *A. caulinodans* ORS571 contains a unique chemotaxis gene cluster (*che*) that includes five chemotaxis genes: *cheAWY1BR* (AZC_0661 to AZC_0665). Two additional chemotaxis genes, namely, *cheY2* (AZC_0620) and *cheZ* (AZC_0621), are located in the distal region of the *che* gene cluster. The *che* gene cluster or *cheA* of *A. caulinodans* controls flagellum-driven motility and chemotactic behavior (18). CheZ also plays an important role in the chemotaxis of *A. caulinodans* (29). However, the contributions of the two CheYs in *A. caulinodans* remain unclear. To elucidate the roles of CheY1 and CheY2, we constructed *cheY1* and *cheY2* gene deletion mutants and studied their regulatory functions.

RESULTS

***A. caulinodans* ORS571 harbors two CheY response regulators.** Genome analysis revealed that the *cheY1* gene (AZC_0663) is located in the chemotaxis gene cluster *cheAWY1BR* (AZC_0661 to AZC_0665) and transcribed in the same direction (Fig. 1). However, the *cheY2* gene (AZC_0620) is located 37 kb upstream of the *cheA* gene and is transcribed in the opposite direction of the downstream chemotaxis gene *cheZ* (Fig. 1). Furthermore, amino acid sequence analysis of CheY1 and CheY2 of *A. caulinodans* and other homologs, the functions of which have been well studied in many organisms, was performed. The amino acid sequence identities of CheY1 and CheY2 were 27.8% to 38.3 and 32.8% to 68.8%, respectively, compared with that of the well-studied CheYs (see Table S1 in the supplemental material). Multiple-sequence alignment results showed that six highly conserved amino acids corresponding to Asp12, Asp13, Asp57, Gly65, Thr87, and Lys109 from *E. coli* CheY were found in both CheY1 and CheY2 (see Fig. S1). Interestingly, the T87+1 residues were all "A" in *E. coli* CheY, *S. meliloti* CheY2, *A. brasilense* CheY4, and *Salmonella enterica* CheY (Fig. S1), which have been demonstrated to control flagellum-mediated chemotaxis (3, 23, 26). We found that the residue at T87+1 was "A" in CheY2 (Fig. S1). These results suggested that CheY2 shares higher amino acid sequence identity with other CheYs than CheY1 and contains an important T87+1 site.

Both CheY1 and CheY2 play roles in chemotaxis of *A. caulinodans* ORS571. To determine the chemotactic roles of *cheY1* and *cheY2* in *A. caulinodans* ORS571, we constructed markerless gene deletion mutants of *cheY1*, *cheY2* (see Fig. S2A and B), and *cheY1-cheY2*. The chemotaxis toward various carbon sources known to be attractants for *A. caulinodans* ORS571 was tested on swim plates (0.3% agar). Representative swim plates are shown in Fig. 2A. The $\Delta cheY1$ mutant formed significantly smaller (55% to 63%) chemotaxis rings than the wild-type (WT) strain on succinate, sodium lactate, and proline plates. However, chemotactic migration of the $\Delta cheY2$ mutant was completely impaired, and this phenotype was similar to those of the $\Delta cheY1 \Delta cheY2$ double mutant, the $\Delta cheA$ mutant (as nonchemotaxis control), and the $\Delta fliM$ mutant (as nonmotile control) (Fig. 2A and B). These results suggest that CheY2 is required for the swimming behavior of *A. caulinodans* ORS571. In addition, the defect of the $\Delta cheY1$ and $\Delta cheY2$ mutants on swim plates was restored to the wild-type (WT) phenotype by

FIG 2 Chemotaxis behavior of *A. caulinodans* ORS571 and $\Delta cheY1$, $\Delta cheY2$, and $\Delta cheY1 \Delta cheY2$ mutants. (A) Swim tests of the wild-type (WT), mutants, and complemented strains ($\Delta cheY1C$ and $\Delta cheY2C$) on soft agar plates. The $\Delta cheA$ and $\Delta flhM$ mutants were used as control mutant strains. The soft agar plates contained 10 mM succinate, sodium lactate, or proline as the carbon source and with 10 mM ammonium chloride as the nitrogen source. The plates with succinate as sole carbon source were used as representative plates. (B) The chemotactic ring diameters were measured for each strain. Average diameters are expressed as percentages relative to that of the WT (defined as 100%). *, $P < 0.05$ versus the WT strain.

complementation (Fig. 2A and B). In addition, there was no growth defect in the mutants compared to growth of the WT cells (see Fig. S3). Therefore, these data suggest that the diminished diameters of the chemotactic rings in different carbon source swim plates were directly related to the *cheY1* and *cheY2* genes.

A competitive capillary assay has previously been used to quantitatively assess the chemotactic and motility behaviors of WT and mutants (18). Figure 3 shows capillaries

FIG 3 Competitive quantitative capillary assays. Statistical analysis of the WT, $\Delta cheY1$, $\Delta cheY2$, and $\Delta cheY1 \Delta cheY2$ cell ratios in the capillary filled with buffer (left), succinate (middle), or sodium lactate (right) as the carbon source. The $\Delta flhM$ mutant was used as a control mutant strain. The error bars represent the standard deviations (SDs) of data from three independent experiments. *, $P < 0.05$ versus the WT strain; **, $P < 0.01$ versus the WT strain.

FIG 4 Paths of swimming cells of *A. caulinodans* ORS571 (WT), $\Delta cheY1$, and $\Delta cheY2$ strains grown in TY medium. Tracks of free-swimming cells were recorded by an Olympus DP73 digital microscope camera. Computerized motion analysis was performed using ICY software. Representative tracks are shown. Each of these trajectories represents 2 s of swimming behavior. Each strain was tested in at least five biological replicates.

filled with buffer as a control and with succinate or sodium lactate as the attractants. When capillaries were filled with buffer, the cell ratio of the $\Delta cheY1$ mutant in the capillaries was less than that of the WT (approximately 40%:60%); however, the cell numbers of $\Delta cheY2$, $\Delta cheY1 \Delta cheY2$, and $\Delta fliM$ mutants were significantly less than that of the WT (Fig. 3). This observation indicated that the motility behavior of the free-swimming cells was affected in the $\Delta cheY1$ and $\Delta cheY2$ mutants. When capillaries were filled with succinate or sodium lactate, the cell number of the $\Delta cheY1$ mutant group was similar to that of the buffer group, but the cell numbers of $\Delta cheY2$, $\Delta cheY1 \Delta cheY2$, and $\Delta fliM$ mutants were less than that of the buffer group (approximately 9% to 10%). This result indicated that CheY2 affected the swimming behavior of the free-swimming cells. The defects of motility and chemotaxis in $\Delta cheY1$ and $\Delta cheY2$ mutants were restored to the WT phenotype by complementation (Fig. 3). Taken together, these results suggested that the motility and chemotaxis behavior of the free-swimming cells were impaired in the $\Delta cheY1$ and $\Delta cheY2$ mutants and that CheY2 is required for chemotaxis behavior in *A. caulinodans* ORS571.

CheY2 mediated the swimming motility bias. In *E. coli*, the flagellar rotary motor turns clockwise when interacting with CheY-P. The phosphorylation state of CheY determines whether cells run or tumble. To evaluate the function of CheY1 and CheY2 in the motility behavior of free-swimming cells, cell swimming paths were observed and analyzed. Cells of *A. caulinodans* ORS571 swim steadily forward (run) and frequently change swimming direction (tumble) (Fig. 4, left). The swimming speed of the $\Delta cheY1$ mutant was similar to that of the WT, but the frequency with which the direction changed was lower than that of the WT cells (Fig. 4, middle). In contrast, the $\Delta cheY2$ mutant cells appeared to change direction much more frequently than the WT but did not exhibit normal smooth swimming (run) (Fig. 4, right). The results suggested that CheY1 and CheY2 affected the frequency with which the direction changed in different ways in *A. caulinodans* and that CheY2 is the major regulator of the swimming motility bias.

CheY1 and CheY2 regulated exopolysaccharide biosynthesis. A previous study suggested that the chemotaxis gene cluster (*cheA* to *cheR*), *cheA*, and *fliM* are involved in exopolysaccharide (EPS) production by *A. caulinodans* ORS571 (18). To determine the role of CheY1 and CheY2 in EPS biosynthesis, the EPS production ability of the $\Delta cheY1$, $\Delta cheY2$, and $\Delta cheY1 \Delta cheY2$ mutants was compared with that of the WT strain. Figure S4 shows that the $\Delta cheY1$ and $\Delta cheY2$ mutants produced less total EPS than the WT on sodium lactate, succinate, or proline as a carbon source. Importantly, the $\Delta cheY2$ mutant had more significant EPS defects than the $\Delta cheY1$ mutant that were similar to those of the $\Delta cheY1 \Delta cheY2$ and $\Delta eps1$ mutants on all tested carbon source plates. Quantitative results further demonstrated these visual observations (Fig. 5). These data indicate that CheY1 and CheY2 are involved in EPS production and that CheY2 has a more significant phenotype than CheY1.

CheY2 is essential for competitive root colonization and nodule occupation with the host. To determine the role of CheY1 and CheY2 in the symbiosis with the

FIG 5 Quantization analysis of extracellular polysaccharide (EPS) production of the wild type and $\Delta cheY1$, $\Delta cheY2$, $\Delta cheY1 \Delta cheY2$, and $\Delta eps1$ mutants on plates with 10 mM indicated carbon sources (sodium lactate, succinate, or proline) and with 10 mM ammonium chloride as a nitrogen source. The error bars indicate SDs from the means of three independent experiments. *, $P < 0.05$ versus the WT strain; **, $P < 0.01$ versus the WT strain.

host *S. rostrata*, the competitive colonization ability on the surface of roots was assayed. The cultures of the WT and the $\Delta cheY1$ or $\Delta cheY2$ mutant were mixed at a ratio of 1:1 and coinoculated with surface-sterilized seedlings. Then, the numbers of WT and mutant cells that were reisolated from the root surfaces of seedlings were taken as the colonization efficiency. The results indicated that the colonization efficiency of the $\Delta cheY1$ mutant was significantly reduced ($P < 0.05$) compared to that of the WT strain (Fig. 6A). Moreover, there was a very significant difference ($P < 0.01$) in the colonization

FIG 6 Analysis of colonization and nodulation phenotypes. (A) Competitive colonization level on the root surface. Bacteria were reisolated from seedlings that were inoculated by the mixed cultures (WT and mutant or complemented strains at a ratio of approximately 1:1) and were detected by PCR. *, $P < 0.05$ versus the WT strain; **, $P < 0.01$. (B) Root nodules induced by WT, $\Delta cheY1$, and $\Delta cheY2$ strains 30 days after inoculation. (C) Stem nodules induced by WT, $\Delta cheY1$, and $\Delta cheY2$ strains 30 days after inoculation. Leghemoglobin of stem nodules shows characteristic orange-brown color. (D) Competitive nodulation tests. Bacteria were reisolated from roots or stem nodules that had been inoculated by the mixed cultures (WT and mutant or complemented strains at a ratio of approximately 1:1) and were detected by PCR. The error bars represent the standard deviations from three independent experiments. *, $P < 0.05$ versus the WT strain; **, $P < 0.01$.

efficiency between the $\Delta cheY2$ mutant and the WT. In addition, we also detected the competitive colonization ability between the WT and $\Delta cheY1 \Delta cheY2$ double mutant. Importantly, the competitive colonization ability of the double mutant was similar to that of the $\Delta cheY2$ mutant. The defect in the competitive colonization ability of the $\Delta cheY1$ and $\Delta cheY2$ mutants was restored to the WT phenotype by complementation (Fig. 6A). These data suggest that CheY2 mainly influences the competitive colonization of host root surfaces.

Generally, defects in competitive root colonization ability should result in defective competitive nodulation ability in the $\Delta cheY1$ and $\Delta cheY2$ mutants. First, we determined the nodulation ability of the WT and mutants. When WT, $\Delta cheY1$, or $\Delta cheY2$ cells were used to individually inoculate the roots or stems of *S. rostrata*, the nodulation ability induced by either mutant was not different from that of the WT (Fig. 6B and C). In addition, leghemoglobin in the stem nodules induced by the $\Delta cheY1$ or $\Delta cheY2$ mutant showed a normal orange-brown color 30 days postinoculation (Fig. 6C). Furthermore, we compared the competitive nodulation ability of the WT and mutants quantitatively. The cultures of the WT and mutants were mixed at a ratio of 1:1 and incubated with roots or stems of *S. rostrata*. Nodulation efficiency was analyzed by counting the numbers of cells of the WT and mutants that were reisolated from the root and stem nodules after incubation for 30 days. As expected, the number of $\Delta cheY1$ or $\Delta cheY2$ cells was significantly reduced compared to the WT cell number (Fig. 6D). However, the nodulation efficiency of the $\Delta cheY2$ mutant revealed more severe defects than that of the $\Delta cheY1$ mutant strain. The defect of competitive nodule occupation in the $\Delta cheY1$ and $\Delta cheY2$ mutants was restored to the WT phenotype by complementation (Fig. 6D). These results showed that $\Delta cheY1$ and $\Delta cheY2$ mutants had an impaired ability to induce host competitive nodule occupation compared to that of the WT but were able to form normal nodules on the roots and stems of the host plant. Taken together, the results show that CheY2 mainly influences competitive root colonization and nodule occupation with the host.

DISCUSSION

Different functions of CheY1 and CheY2 in chemotaxis and motility. Comparative genome analyses of chemotaxis in diverse motile bacteria showed that most bacteria encode two or more chemotaxis systems (15). Many species have multiple copies of a single gene. In some species, multiple CheYs perform different functions. For example, the *A. brasilense* Che1 pathway regulates changes in swimming speed, and Che4 regulates the probability of swimming reversal (26). In *S. meliloti*, the effect of CheY2-P on flagellum rotation is regulated by CheY1, which may be caused by the competition of CheA with phosphate (24). In this study, two *cheY* genes from *A. caulinodans* ORS571 were studied. By comparing the biological characteristics between the mutants and the WT strain, we significantly improved our understanding of the function of the two CheYs. Based on the results of the swim plate and competitive capillary assays, we found that the phenotype of the $\Delta cheY2$ mutant was similar to those of the $\Delta cheY1 \Delta cheY2$ double mutant, the nonmotile $\Delta fliM$ mutant, and the $\Delta cheA$ (Fig. 2) and $\Delta cheZ$ (29) chemotaxis mutants of *A. caulinodans* ORS571. This result revealed a critical role of CheY2 in motility and chemotaxis. CheY1 was also important, although its effects were more modest. In addition, the motility behavior of free-swimming cells (Fig. 4) suggested that the $\Delta cheY2$ mutant changed direction much more frequently than the WT parent. However, the frequency of the direction change in the $\Delta cheY1$ mutant was lower than that of the WT cells. The results suggested that CheY1 and CheY2 affect the motility frequency in different ways in *A. caulinodans*.

CheY1 and CheY2 regulate EPS biosynthesis. Most chemotaxis pathways found in bacterial genomes are predicted to regulate flagellum-mediated motility patterns. In addition to contributing to chemotactic and motility behavior, the response regulators CheY1 and CheY2 appear to be involved in EPS biosynthesis. We found that the $\Delta cheY1$, $\Delta cheY2$, and $\Delta cheY1 \Delta cheY2$ mutants produced less total EPS than the WT under all tested conditions and that the $\Delta cheY2$ mutant had more significant defects than the

$\Delta cheY1$ mutant. These data indicate that CheY1 and CheY2 are involved in EPS production. A previous study suggested that the CheA-R, chemotaxis protein CheA, and flagellar motor protein FlhM have positive regulatory roles in EPS production in *A. caulinodans* ORS571 (18) and that CheZ has a negative regulatory role in EPS production (29). Similarly, CheY2 also positively regulates the chemotactic response and EPS production. Interestingly, the $\Delta cheY1$ mutation, which mediated approximately 40% of the WT chemotactic behavior, also regulated EPS production compared to that in the WT strain. These results suggested that chemotaxis response regulators CheY1 and CheY2 also play important roles in EPS production. Further studies are needed to determine the mechanism underlying the role of chemotaxis and motility in the regulation of the EPS biosynthesis.

CheY1 and CheY2 are involved in competitive colonization with the host. Chemotaxis signals allow motile soil bacteria to sense and respond to compounds released by plant roots. Chemotaxis provides bacteria with a competitive advantage in the colonization of plant root surfaces (30). In addition, motility is a characteristic of rhizobia that can contribute to the competitive symbiotic success of these bacteria (31). The competitive colonization and nodule occupation experiments clearly demonstrated that CheY2 promotes competitive nodule occupation in the host plant. In addition, the $\Delta cheY1$ mutant is important in competitive nodule occupation. The competitiveness for nodule occupation with the host was similar between the CheY2 and CheA (18) or CheZ of *A. caulinodans* ORS571 (29). When the WT and mutants were used to individually inoculate the roots or stems of *S. rostrata*, nodulation showed normal leghemoglobin induction in host plants (Fig. 6C). The leghemoglobin phenotype was different from that of a *ggm* mutant in that leghemoglobin induction in host plants did not occur in the mutant nodules (32). One implication of these results is that the signaling compounds of the rhizosphere directly trigger chemotaxis of the motile *A. caulinodans* cells in soil and facilitate colonization and nodulation on roots. Another possible explanation is that EPS production by *A. caulinodans* cells promotes root colonization and nodulation.

Taken together, the results in this study expand the horizon of chemotaxis response regulator CheYs in *Alphaproteobacteria*. However, more studies are required to help further elucidate the molecular mechanisms of CheYs in chemotaxis and EPS production.

MATERIALS AND METHODS

Strains and growth conditions. All strains and plasmids used in this study are shown in Table 1. *E. coli* was cultured overnight in LB medium at 37°C and supplemented with antibiotics when necessary. WT strain *A. caulinodans* ORS571 and mutant strains were grown in L3 (33) or TY (5 g liter⁻¹ tryptone, 3 g liter⁻¹ yeast extract, and 0.83 g liter⁻¹ CaCl₂·2H₂O) medium containing 100 μg ml⁻¹ ampicillin and 25 μg ml⁻¹ nalidixic acid antibiotics at 37°C. The L3 minimal medium was supplemented with 10 mM NH₄Cl.

Construction of mutant and complemented strains. To construct the plasmid that was used for deletion of the *cheY1* (AZC_0663) gene, an 811-bp upstream fragment (UF) was amplified using the primer pair cheY1UF/cheY1UR (Table 2), and a 903-bp downstream fragment (DF) was amplified by PCR using the primers cheY1DF/cheY1DR. Genomic DNA isolated from *A. caulinodans* ORS571 was used as a template. The UF was digested with KpnI-NdeI and ligated into the allelic exchange vector pCM351 (34). The resulting recombinant plasmid was designated pCM351::UF1. The DF was digested with Apal-AgeI and cloned into the recombinant plasmid pCM351::UF1. The recombinant plasmid pCM351::UF1::DF1 was transferred into *A. caulinodans* ORS571 by triparental conjugation with the helper plasmid pRK2013 (35). Recombinants from double homologous recombination were obtained and screened on TY plates by selecting for gentamicin resistance and tetracycline sensitivity (34). The potential mutant was generated by in-frame deletion of an internal 312-bp fragment and insertion of a gentamicin resistance gene. Then, the gentamicin gene was deleted by introduction of the Cre expression plasmid pCM157 (34). The correct mutation was verified by PCR with the primer pair cheY1F/cheY1R (Table 2; see also Fig. S2C in the supplemental material). The expected 384-bp PCR product was observed in the WT strain, while a smaller product (72 bp) was observed in the $\Delta cheY1$ mutant. To generate the complemented strain of the $\Delta cheY1$ mutant, a fragment containing the entire open reading frame (ORF) and the predicted promoter sequence of *cheA* (601 bp) was amplified from the genomic DNA of ORS571 and ligated. The ligated fragment was digested with BamHI-XbaI and cloned into pBBR-MCS2 (36). The resulting plasmid was conjugated into the $\Delta cheY1$ mutant. The complemented strain was designated $\Delta cheY1C$.

TABLE 1 Strains and plasmids used in this study

Strain or plasmid	Relevant properties ^a	Source or reference
Strains		
<i>A. caulinodans</i> ORS571	Wild-type strain, Amp ^r , Nal ^r	28
Δ <i>cheY1</i> mutant	ORS571 derivative, <i>cheY1</i> gene deletion mutant, Amp ^r , Nal ^r	This study
Δ <i>cheY2</i> mutant	ORS571 derivative, <i>cheY2</i> gene deletion mutant, Amp ^r , Nal ^r	This study
Δ <i>cheY1</i> Δ <i>cheY2</i> mutant	ORS571 derivative, <i>cheY1</i> and <i>cheY2</i> gene deletion mutant, Amp ^r , Nal ^r	This study
Δ <i>eps1</i> mutant	ORS571 derivative, <i>eps1</i> gene cluster deletion mutant, Amp ^r , Nal ^r	This study
Δ <i>cheA</i> mutant	ORS571 derivative, <i>cheA</i> deletion mutant, Amp ^r , Nal ^r	18
Δ <i>flhM</i> mutant	ORS571 derivative, <i>flhM</i> deletion mutant, Amp ^r , Nal ^r	18
Δ <i>cheY1C</i> mutant	ORS571 derivative, <i>cheY1</i> deletion mutant carrying pBBR1MCS-2- <i>cheY1</i> , Amp ^r , Nal ^r , Kan ^r	This study
Δ <i>cheY2C</i> mutant	ORS571 derivative, <i>cheY2</i> deletion mutant carrying pBBR1MCS-2- <i>cheY2</i> , Amp ^r , Nal ^r , Kan ^r	This study
WT-pBBR	Wild-type strain carrying pBBR1MCS-2, Amp ^r , Nal ^r , Kan ^r	18
<i>E. coli</i> DH5 α	F ⁻ <i>supE44</i> Δ <i>lacU169</i> (ϕ 80 <i>lacZ</i> M15) <i>hsdR17</i> <i>recA1</i> <i>endA1</i> <i>gyrA96</i> <i>thi-1</i> <i>relA1</i>	Transgen
Plasmids		
pCM351	Mobilizable allelic exchange vector, Amp ^r , Gen ^r	34
pRK2013	Helper plasmid, ColE1 replicon, Tra ⁺ , Kan ^r	35
pCM157	IncP plasmid that expresses Cre recombinase, Tet ^r	34
pBBR1MCS-2	Broad-host-range plasmid, Kan ^r	36
pBBR- <i>cheY1</i>	pBBR1MCS-2 carrying the native promoter of the <i>cheA</i> and <i>cheY1</i> genes, Kan ^r	This study
pBBR- <i>cheY2</i>	pBBR1MCS-2 carrying the native promoter of the <i>cheY2</i> gene, Kan ^r	This study

^aAmp^r, ampicillin resistance; Nal^r, nalidixic acid resistance; Gen^r, gentamicin resistance; Kan^r, kanamycin resistance; Tet^r, tetracycline resistance.

To construct the plasmid that was used for the *cheY2* (AZC_0620) gene deletion, a 610-bp UF was amplified using primers *cheY2UF* and *cheY2UR*, and a 624-bp DF was amplified by PCR using primers *cheY2DF* and *cheY2DR*. The UF was digested with KpnI-NdeI and ligated into pCM351. The resulting recombinant plasmid was designated pCM351::UF2. The DF was digested with Apal-AgeI and cloned into the recombinant plasmid pCM351::UF2. The PCR product corresponding to the DF was cloned into pCM351::UF2. The recombinant plasmid was transferred into ORS571. The potential mutant was generated by in-frame deletion of an internal 324-bp fragment and was verified by PCR with the primer pair *cheY2F* and *cheY2R* (Table 2; Fig. S2D). The PCR product of the WT amplified with primer pair *cheY2F*/*cheY2R* was 384 bp in length, while that of the *cheY2* mutant was 62 bp, and a confirmed mutant was named Δ *cheY2*. To generate the complemented strain of the Δ *cheY2* mutant, a fragment containing the entire ORF and the 324-bp sequence immediately upstream (predicted promoter) of *cheY2* was amplified. The fragment was digested with BamHI-XbaI and cloned into pBBR-MCS2. The resulting plasmid was conjugated into the Δ *cheY2* mutant. The complemented strain was designated Δ *cheY2C*.

A Δ *cheY1* Δ *cheY2* double mutant resulting from a transfer of the recombinant plasmid pCM351::UF1::DF1 was transferred into the Δ *cheY2* mutant by triparental conjugation with the helper plasmid pRK2013 for homologous recombination exchange. Recombinants from double homologous recombination were obtained and screened on TY plates by selecting for gentamicin resistance and tetracycline

TABLE 2 Primers used in this study

Primer	Sequence (5'→3') ^a	Purpose
<i>cheY1UF</i> -KpnI	GGGGTACCGATCATCGCCTTGTCGAGC	Δ <i>cheY1</i> construction
<i>cheY1UR</i> -NdeI	GGAATTCATATGATCCACCACTAGGCAGGTC	Δ <i>cheY1</i> construction
<i>cheY1DF</i> -Apal	CGGGGCCCGAGAAGCTTGAGGAAGTCG	Δ <i>cheY1</i> construction
<i>cheY1DR</i> -AgeI	GACCGGTGCATGTGCTGCACGATCAC	Δ <i>cheY1</i> construction
<i>cheY2UF</i> -KpnI	GGGGTACCCAAGCTTCATCCGATCAGG	Δ <i>cheY2</i> construction
<i>cheY2UR</i> -NdeI	GGAATTCATATGTTCAATGCGCAGACGCTG	Δ <i>cheY2</i> construction
<i>cheY2DF</i> -Apal	CGGGGCCCGAACCAGGCTTGTCAGGTC	Δ <i>cheY2</i> construction
<i>cheY2DR</i> -AgeI	GACCGGTGAGCGTGGCGATGGAATTG	Δ <i>cheY2</i> construction
<i>eps1UF</i> -KpnI	GGGGTACCTACATCTCGTGGCGCATCC	Δ <i>eps1</i> construction
<i>eps1UR</i> -NdeI	GGAATTCATATGTGCAGATCCTCGAGCAGC	Δ <i>eps1</i> construction
<i>eps1DF</i> -AgeI	GACCGGTGTGGTCCAGCGTCTCCG	Δ <i>eps1</i> construction
<i>eps1DR</i> -SacI	CGAGCTCGCGCTGATCGAGCAGGCC	Δ <i>eps1</i> construction
<i>cheY1CF</i> -SpeI	GACTAGTTGGCGAGCGAAGTGAAGTC	Δ <i>cheY1C</i> construction
<i>cheY1CR</i> -XbaI	GCTCTAGAGTCGAAGTGGCGATGTAGT	Δ <i>cheY1C</i> construction
<i>cheY2CF</i> -SpeI	GACTAGTCCTCGAGTTCATTGAGAA	Δ <i>cheY2C</i> construction
<i>cheY2CR</i> -XbaI	GCTCTAGATCAGGCCTCGAACACGGT	Δ <i>cheY2C</i> construction
<i>cheY1F</i>	ATGAAGACCTGCCTAGTGGTG	Validation of <i>cheY1</i>
<i>cheY1R</i>	TCATGTAGCGGGTGCCTTCTC	Validation of <i>cheY1</i>
<i>cheY2F</i>	ATGGCGGTTGACCTGACAATG	Validation of <i>cheY2</i>
<i>cheY2R</i>	TCAGGCCTCGAACACGGTGTG	Validation of <i>cheY2</i>

^aEngineered restriction sites are underlined.

sensitivity. PCR was used to verify the potential double mutant, and one such strain was a $\Delta cheY1 \Delta cheY2$ double mutant.

To construct the plasmid that was used for the *eps1* (AZC_1831 to AZC_1834) gene cluster deletion, a 750-bp UF was amplified using the primer pair *eps1UF/eps1UR*, and a 789-bp DF was amplified by PCR using the primer pair *eps1DF/eps1DR*. The UF was digested with KpnI-NdeI and ligated into pCM351. The resulting recombinant plasmid was designated pCM351::UF. The DF was digested with AgeI-SacI and cloned into the recombinant plasmid pCM351::UF. The PCR product corresponding to the DF was cloned into pCM351::UF. The recombinant plasmid pCM351::UF:DF was transferred into ORS571. The potential mutant was generated by in-frame deletion of an internal 3,223-bp fragment, and a $\Delta eps1$ mutant was confirmed.

Chemotaxis assays. The chemotactic responses of the WT and mutant strains were detected on soft agar plates. The swimming behavior was analyzed in plates with L3 medium containing 0.3% agar. To compare the chemotactic responses to different carbon sources, 10 mM succinate, sodium lactate, or proline as the sole attractant was added to the L3 medium, and 10 mM NH_4Cl was added as the nitrogen source. These plates were inoculated with the WT and mutants as described previously (18). The swimming diameters were recorded after incubation for 48 to 72 h at 37°C.

Quantitative capillary chemotaxis assays were performed as previously described by Liu et al. (18) with a few modifications. Briefly, overnight cultures were adjusted to an optical density at 600 nm (OD_{600}) of 1.0, and 300- μl aliquots of cell suspensions (WT/mutants mixed at 1:1) were added to a 96-well plate. Capillaries were filled with 10 mM succinate or sodium lactate as an attractant or with chemotaxis buffer as a control. Then, these capillaries containing attractant or buffer were placed in a bacterial suspension in a 96-well plate. After incubation for 1 h at 37°C, the liquid (containing bacteria) in the capillaries was then pipetted into 1 ml of chemotaxis buffer, and serial 10-fold dilutions were placed on TY plates containing nalidixic acid and ampicillin. When the WT was mixed with the mutant at 1:1, the WT and mutant with the same resistance could not be distinguished by resistance after being reisolated from capillaries. Colonies (containing WT and mutant) on TY plates (containing ampicillin and nalidixic acid) were further identified by PCR using the primer pair *cheY1F/cheY1R* or *cheY2F/cheY2R* to detect the *cheY1* or *cheY2* gene, respectively, and distinguish the WT and mutants. To minimize this systematic error, we optimized the experimental conditions to obtain reproducible data, and the number of cells detected in capillaries was approximately 1,000 (96-well plate $\times$ 10).

To observe the motility behavior of the mutants, cells were grown in TY medium to mid-exponential phase, and the motility behavior was observed and recorded by an Olympus DP73 digital microscope camera. For each strain, the swimming path of at least 50 cells was tracked manually by ICY software in the video recording (37). The cell paths were analyzed for each strain for 2 s, and at least 5 independent experiments were observed.

EPS production. To test the production of EPS, overnight bacterial cultures were adjusted to an OD_{600} of 1.0. Then, 15 μl of the bacterial suspension was inoculated onto 0.8% solid L3 plates (with NH_4Cl) for 3 days at 37°C. EPS production was quantified based on the method described by Nakajima et al. (38) with some modifications. Bacteria on the L3 plates were collected and resuspended in λ -buffer (10 mM Tris-HCl [pH 7.0], 10 mM MgSO_4). The supernatant containing soluble EPS components was treated with concentrated sulfuric acid with 0.2% anthrone, incubated for 7 min at 100°C, and then chilled on ice. The OD_{620} of the chilled mixture was measured using a NanoDrop 2000C spectrophotometer. The EPS content was evaluated by normalizing the collected cell suspensions to the OD_{600} . D-Glucose was used for preparation of the standard curve.

Colonization assay. The colonization assay was performed according to a previously described method by Liu et al. (18) with a few modifications. The seeds of *S. rostrata* were treated with concentrated sulfuric acid for 25 min, washed with sterile water 5 times, and soaked in sterile water on trays at 30°C in the dark for 2 to 3 days. Overnight bacterial cultures were adjusted to an OD_{600} of 1.0. The WT and mutants were mixed at 1:1 and added to 600-ml bottles containing 200 ml of molten semisolid agar (0.3% agar). Ten seedlings (approximately 2 cm in length) were transferred into the bottles and grown at 28°C. Seven days after inoculation, the roots were washed and homogenized by a homogenizer. Serial dilutions of the supernatant were spread on TY medium agar plates containing ampicillin and nalidixic acid (on which WT, mutant, and complemented strains can grow) or containing ampicillin, nalidixic acid, and kanamycin (on which only complemented strains can grow). The competitive colonization ratio between the WT and complemented strains was determined by counting the numbers of cells on the TY antibiotic plates. The competitive colonization efficiency between WT and $\Delta cheY1$ or $\Delta cheY2$ colonies on TY plates, which cannot be distinguished by resistance, was identified by PCR using the primers *cheY1F/cheY1R* and *cheY2F/cheY2R* to distinguish the WT and mutant strains.

Nodulation and competitive nodulation assays. Nodulation and competitive nodulation assays were carried out as described previously (39) with some modifications. For the nodulation assay, three treatments were used: WT alone, $\Delta cheY1$ alone, and $\Delta cheY2$ alone. For the competitive nodulation assay on roots or stems, four treatments were used: WT/ $\Delta cheY1$ mixed at 1:1; WT/ $\Delta cheY2$ mixed at 1:1; WT/ $\Delta cheY1C$ mixed at 1:1, and WT/ $\Delta cheY2C$ mixed at 1:1. For the nodulation assays on roots, surface-sterilized seedlings were soaked in cell suspensions from each treatment for 30 min. Then, the seedlings were transferred to vermiculite in moistened pots containing a low-N nutrient (40). For nodulation assays on stems, *S. rostrata* seeds were germinated and transferred to vermiculite in moistened pots containing a low-N nutrient. After 30 days of plant growth, *S. rostrata* stems were inoculated with cell suspensions from each treatment at an OD_{600} of 0.5. All *S. rostrata* plants were grown at 28°C in a greenhouse. Nodules were harvested 30 days postinoculation and counted. For competitive nodulation, bacteria were reisolated from surface-sterilized roots or stem nodules. Nodules were collected, surface-sterilized,

crushed, and plated on TY agar plates that contained ampicillin and nalidixic acid or ampicillin, nalidixic acid, and kanamycin. The competitive nodulation experiment between the WT and complemented $\Delta cheY1C$ or $\Delta cheY2C$ strains was determined by counting the numbers of cells on the TY antibiotic plates containing ampicillin and nalidixic acid with or without kanamycin. The competitive nodulation efficiency between the WT and $\Delta cheY1$ or $\Delta cheY2$ mutant was analyzed by determining the numbers of clones on the plates by PCR using the primer pair *cheY1F/cheY1R* or *cheY2F/cheY2R*.

Phylogenetic analyses. Protein sequences were grouped and aligned using the ClustalW module of BioEdit software (41). Phylogenetic analyses were performed by using the neighbor-joining (NJ) method with 1,000 bootstrap replications. The analysis involved nine amino acid sequences, namely, CheY1 (BAF86661) and CheY2 (BAF86618) of *A. caulinodans*, CheY (WP_000763860) of *E. coli*, CheY1 (WP_003529950) and CheY2 (WP_003529938) of *S. meliloti*, CheY1 (WP_035678531) and CheY4 (WP_035670599) of *A. brasilense*, CheY of *S. enterica* (WP_000763861), and CheY1 (WP_000772151) of *Helicobacter pylori* (42).

Statistical analysis. To compare the WT and mutant phenotypes (chemotaxis, EPS quantitation, plant colonization, and nodule occupation assays), all statistical analyses were performed using the SPSS 17.0 software package. We determined average values from at least three independent experiments and performed one-way analysis of variance, followed by pairwise two-sample *t* tests assuming equal variances. Student's *t* test assuming equal variances was used to calculate the *P* values. *P* values of <0.05 and <0.01 were considered significant. Each experiment was repeated at least three times.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 0.4 MB.

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Xiaoke Hu and Wei Liu designed the experiments. Wei Liu, Xue Bai, Yan Li, and Yachao Kong performed the experiments. Wei Liu, Xiaoke Hu, and Jun Min analyzed data and wrote the paper. All authors contributed to the manuscript revisions.

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