Inactivation of the Phosphatase CheZ Alters Cell-Surface Properties of *Azorhizobium caulinodans* ORS571 and Symbiotic Association with *Sesbania rostrata*

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Azorhizobium caulinodans can form root and stem nodules with the host plant *Sesbania rostrata*. The role of the CheZ phosphatase in the *A. caulinodans* chemotaxis pathway was previously explored using the nonchemotactic *cheZ* mutant strain (AC601). This mutant displayed stronger attachment to the root surface, enhancing early colonization; however, this did not result in increased nodulation efficiency. In this study, we further investigated the role of CheZ in the interaction between strain ORS571 and the roots of its host plant. By tracking long-term colonization dynamic of *cheZ* mutant marked with LacZ, we found a decrease of colonization of the *cheZ* mutant during this process. Furthermore, the *cheZ* mutant could not spread on the root surface freely and was gradually outcompeted by the wild type in original colonization sites. Quantitative reverse-transcription PCR analyses showed that *exp* genes encoding exopolysaccharides synthesis, including *oac3*, were highly expressed in the *cheZ* mutant. Construction of a strain carrying a deletion of both *cheZ* and *oac3* resulted in a mutant strain defective in the colonization process to the same extent as found with the *oac3* single-mutant strain. This result suggested that the enhanced colonization of the *cheZ* mutant may be achieved through regulating the formation of exopolysaccharides. This shows the importance of the chemotactic proteins in the interaction between rhizobia and host plants, and expands our understanding of the symbiosis interaction between rhizobium and host plant.

Keywords: genetics and gene regulation, rhizobium–legume symbiosis

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Rhizobia can fix atmospheric nitrogen and promote plant growth and, in turn, plants can exude various carbon and nitrogen sources, including sugar, amino acids, organic acids, and other compounds which provide nutrition and a suitable niche for rhizobia (Kamilova et al. 2006).

Azorhizobium caulinodans ORS571 was isolated from the stem of the tropical legume *Sesbania rostrata* (Dreyfus et al. 1988). This strain can induce nitrogen-fixing nodules on both the roots and the stems of the host. *A. caulinodans* can also fix nitrogen in a free-living state. Nodule formation of stems proceeds via crack entry at sites corresponding to predetermined primordia (Fernández-López et al. 1998; Tsien et al. 1983). For root nodulation, two modes of infection are employed by *A. caulinodans*: root hair invasion and intercellular invasion at the lateral root base. Root hair invasion is the best-known mode of invasion used in many model legumes such as *Lotus japonica* and *Medicago truncatula* (Goormachtig et al. 2004), while crack entry infection at lateral root base is found in some tropical legumes, including *Arachis hypogae* and *Neptunia* sp. (Chandler 1978; Rivas et al. 2002). In the case of *S. rostrata*, which is an aquatic legume, an infection route through root hair curling by *Azorhizobium caulinodans* occurs under nonaquatic conditions, while infection by intercellular invasion occurs under flooded conditions. In addition to the infection modes, *S. rostrata* can form two types of nodules with *A. caulinodans*, determinate and indeterminate (Bomfeti et al. 2013; Ndoye et al. 1994). These two types of nodules can be differentiated by their morphology, physiology, and stage of development (Hirsch 1992). The plant hormone ethylene is a key factor controlling the type of nodules (Fernández-López et al. 1998). In addition to forming nodules with *S. rostrata*, *A. caulinodans* ORS571 could also infect many cereals such as rice and wheat (H. Liu et al. 2017).

The interaction between rhizobia and plants involves the chemotactic attraction of the bacteria toward the plant root system, followed by adsorption, colonization, proliferation, infection, and nodule morphogenesis. Chemotaxis is the first and essential process in the competitive rhizosphere environment (Scharf et al. 2016). In *Escherichia coli*, bacteria move toward the suitable niche via methyl-accepting chemoreceptors sensing extracellular signals. The presence of repellents or absence of attractants could increase the autokinase activity of CheA. CheA phosphorylates the response regulator CheY, and the resulting phosphorylated CheY (CheY-P) regulates motor switching via binding to the flagellar motor protein FliM. CheY-P can be dephosphorylated by its own dephosphorylation ability. An additional phosphatase CheZ can accelerate the dephosphorylation

of CheY-P and terminate the signal (Jones and Armitage 2015). For many bacteria such as *Helicobacter pylori*, *Ralstonia solanacearum*, *Bacillus subtilis*, and *B. amyloliquefaciens*, non-chemotactic mutants are significantly impaired in their colonization and invasion of the host roots (Aihara et al. 2014; Yao and Allen 2006; Yaryura et al. 2008) and, in particular, the initial colonization is impaired (Allard-Massicotte et al. 2016; McGee et al. 2005; Terry et al. 2005). In *A. caulinodans*, when the *che* gene cluster, including *cheA*, *cheW*, *cheY2*, *cheB*, and *cheR*, was deleted, the mutants were defective in competitive colonization and nodulation with host plants (W. Liu et al. 2018). In addition, two chemoreceptors encoded by *icpB* and *tlpA1* also were involved in the symbiosis in *A. caulinodans* (Jiang et al. 2016; W. Liu et al. 2017). Chemotaxis contributes to a competitiveness in the colonization process (Lacal et al. 2010; Vallad and Subbarao 2008). However, in contrast to what was observed for other chemotaxis mutants, early colonization was enhanced by *A. caulinodans* carrying a deletion of an orphan *cheZ* gene (X. Liu et al. 2018), and the mechanism of the unusual function of CheZ remains unknown.

Exopolysaccharides (EPS) play an important role in the establishment of an association between rhizobia and plants. EPS can protect rhizobia against plant defense compounds either as structural components or as signaling molecules (Mathis et al. 2005; Wakao et al. 2015). EPS are essential for the complete nodule development induced by *Rhizobium leguminosarum* CFN42 on legume plants (Cava et al. 1989). The modification of EPS can influence maize root colonization by *Azospirillum brasilense* (Jofré et al. 2004). In addition, a rhamnose-deficient EPS mutant of *Rhizobium* sp. IRBG74 is defective in symbiosis (Mittra et al. 2016). The symbiosis of *Azorhizobium caulinodans* with *S. rostrata* is also influenced by EPS production (Gao et al. 2001; Mathis et al. 2005). The EPS synthesized by *A. caulinodans* is composed of a linear homopolysaccharide made up of α -1,3-linked 4,6-O-(1-carboxyethylidene)-D-galactosyl residues (D'Haeze

et al. 2004). Two EPS biosynthesis-related gene clusters have been identified in the *A. caulinodans* genome (Tsukada et al. 2009). Cluster I is composed of four genes, including *oac3* (*azc_1831*), *oac2* (*azc_1832*), *oac1* (*azc_1833*), and *oac0* (*azc_1834*). Cluster II is composed of 14 genes, from *azc_3319* to *azc_3332* (Nakajima et al. 2012). The *oac3* and *oac2* genes encode a dTDP-D-glucose synthase and a dTDP-L-rhamnose synthase, respectively. The *oac3* and *oac2* disrupted mutants failed to produce EPS and initiate the process of infection and thread formation (Gao et al. 2001; Goethals et al. 1994). However, previous reports are mostly focused on the effect of EPS on the later stages of the interaction between rhizobia and plants, and the role of EPS during early stages of the interaction remains unknown.

In this study, we assessed the role of the *cheZ* mutant in the nodulation process and in long-term colonization. In addition, we found that EPS production might be responsible for the competitive advantages of the *cheZ* mutant strain in the initial colonization compared with the wild type. This study may provide an insight to reconsider the roles of chemotaxis protein in host colonization.

RESULTS

The enhanced competitive colonization of the *cheZ* mutant strain AC601 in the early stages.

We previously reported that CheZ in *A. caulinodans* negatively regulates the process of surface adhesion of bacterial cells on the root system (X. Liu et al. 2018). In the experimental design, previously used surface adhesion was determined after a relatively long time of incubation (4 and 24 h) using inoculum of high cellular density (X. Liu et al. 2018). Therefore, to challenge former conclusions, a new set of experiments was performed using shorter incubation times and lower cell density.

Competitive colonization of the wild-type strain ORS571 and the *cheZ* mutant (strain AC601) with various inoculant

Fig. 1. Early-term competitive colonization of *Sesbania rostrata* seedlings by the *Azorhizobium caulinodans* wild-type and *cheZ*-deletion mutant strains. **A**, Dose response and **B**, time response. Seedlings of *S. rostrata* were inoculated by wild-type strain ORS571 and the *cheZ* mutant strain AC601 mixed at equal concentrations. The relative colonization ratio of the wild type and the *cheZ* mutant was determined after 4 h in a dose response assay, and the initial concentration of bacteria was adjusted to an optical density at 600 nm of 0.6 in a time response assay. Data are means $\pm$ standard deviation from at least three independent experiments. An asterisk (*, $P < 0.01$) indicates a Student's *t* test of the mutant with respect to the wild type. The significant difference between different conditions is shown on the top of the column chart according to a χ^2 analysis performed by SPSS; ns above the bars indicates not statistically different according to the χ^2 analysis.

concentrations of optical density at 600 nm (OD_{600}) of 0.05 to 0.8 was analyzed after 240 min of incubation (Fig. 1A). Whatever the concentration of each strain, when the ratio between the wild type and the *cheZ* mutant was 1:1, quantification of the bacteria attached to the root system showed a higher number for the *cheZ* mutant than for the wild type, suggesting that the *cheZ* mutant had a competitive advantage at the early stages of the colonization process. Then, the experiment was repeated with an initial inoculation concentration of OD_{600} of 0.6 and quantification was performed and stopped at different times after inoculation (Fig. 1B). The number of bacterial cells on the roots increased with the inoculation time; however, the colonization ratios of the wild type and strain AC601 remained almost unchanged. During incubation, the number of strain AC601 mutants that adhered to the root surface of the host was approximately twofold greater than that of the wild type. These results showed that the competitive advantage of strain AC601 was robust and independent of population density and incubation time.

The *cheZ* mutant strain AC601 was outcompeted in competitive nodulation but loss of *cheZ* could not affect effectiveness of nodules.

Chemotaxis is an important factor in the process of competitive nodulation (Fig. 2). In order to investigate whether the enhanced colonization of the *cheZ* mutant could result in an increase of root nodulation, strains ORS571 and AC601 were inoculated at equal concentrations, and the proportion of their final nodule occupancy was analyzed. Because strain AC601 is defective in chemotaxis, the *Sesbania* seedlings were submerged into bacterial culture for 4 h (50 rpm) before being transplanted to Leonard jars filled with vermiculite. Surprisingly, in contrast to the results observed in early colonization experiments, the wild-type strain ORS571 had relatively higher nodule occupancy than strain AC601 in both competitive root nodulation and stem nodulation. To further confirm this result, we compared the relative competitive nodulation ability between the two strains from 10 days postinoculation (dpi) (when the nodules begin to be

formed) to 43 dpi. The nodule number increased with time in strain ORS571; however, the occupancy of strain AC601 had no significant change, indicating that the nodulation ability of strain AC601 was limited under this stage. These results clearly showed that the enhanced colonization of strain AC601 could not result in an increase of the nodule occupancy.

In order to determine whether the defect of strain AC601 on competitive nodulation was caused by a defect in nitrogen fixation, we checked the effectiveness of nodules formed by strain AC601. Both strains ORS571 and AC601 can form root nodules that were pink in color (Supplementary Fig. S1). The pink color of nitrogen-fixing nodules results from the presence of oxygen-sequestering leghemoglobin (Barnett and Long 2018), indicating that the symbiotic properties are not influenced by *CheZ*. In addition, the wild type and *cheZ* mutant, when applied alone, had no significant difference on root nitrogen fixation abilities (Supplementary Fig. S2).

Dynamics of long-term colonization of strain AC601 and the wild type.

Because strain AC601 (*cheZ* mutant) could induce the development of normal nodules, we further determined whether the mutant strain had lost its competitive advantages during the long-term colonization process. The dynamic long-term colonization of strains ORS571 and AC601 on *S. rostrata* roots was monitored. First, strains ORS571 and AC601 were marked with LacZ (pXLGD4) and inoculated separately. Then, the distribution of strains ORS571 and AC601 on roots was tracked at different times. Interestingly, the colonization efficiency (degree of being dyed blue) of *cheZ* mutant strain AC601 was much more than that of the wild type after initial inoculation (4 h) whereas, at 3 dpi, the extent of colonization with strain AC601 or ORS571 was similar (Fig. 3). Over 5 days, the degree of strain ORS571 colonization was much more than that of strain AC601 and, near root tips, the difference between strains ORS571 and AC601 was more significant (Fig. 3).

Fig. 2. Competitive nodulation of the wild type and the *cheZ* mutant. **A**, Root and shoot nodule occupancy of wild-type strain ORS571 and *cheZ* mutant strain AC601. **B**, Dynamics of root nodulation of the wild type and *cheZ* mutant; dpi = days postinoculation. Values are means $\pm$ standard deviation calculated from more than three independent experiments. An asterisk (*) indicates $P < 0.01$ versus the wild type by a Student's *t* test. A χ^2 analysis was performed to show the significant difference between different conditions; ns above the bars indicates not statistically different according to the χ^2 analysis.

In addition, strains ORS571 and AC601 were mixed in equal proportion prior to inoculation, and their relative concentration in long-term competitive colonization (from 4 h to 15 days) along the root surface was analyzed (Fig. 4). In the root base

region (0 to 2 cm), strains AC601 and ORS571 were in equal ratio within 3 days, whereas strain AC601 was outcompeted by strain ORS571 after 5 days. In the fresh elongation region (>3 cm), strain ORS571 showed the most abundant cells

Fig. 3. Dynamics of long-term root surface colonization of wild-type strain ORS571 and *cheZ* mutant strain AC601. For each picture, seedlings inoculated with the wild type are positioned to the left, the *cheZ* mutant strain AC601 is in the middle, and the noninoculated control is on the right. Inoculated strains carry pXLGD4 containing the *lacZ* gene. In situ 5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside staining was performed after **A**, 4 h postinoculation; **B**, 3 days postinoculation (dpi); **C**, 5 dpi; **D**, 7 dpi; and **E**, 10 dpi. Black arrows indicate the sites on the plant with significant difference between plants inoculated with the wild type and the *cheZ* mutant.

colonizing the root surface, and strain AC601 showed gradually decreased occupancy alongside the root surface in the direction of root tip. The number of nodules of the wild type was approximately 60-fold more than that of the *cheZ* mutant in the root region near the tip (4 to 5 cm) at 10 dpi whereas, at the base of the roots, the number of nodules of the wild type was approximately 2.5-fold higher than that of *cheZ* mutant. These results suggested that the competitive advantages of the *cheZ* mutant in early colonization are lost in long-term colonization, and CheZ helps *A. caulinodans* spread alongside the root surface.

Fig. 4. Dynamics of competitive long-term colonization of the wild type and the *cheZ* mutant. In the dynamic of competitive long-term colonization, wild-type strain ORS571 and *cheZ* mutant strain AC601 were inoculated in equal proportions. The distance from the site of the nodule to the base of the root is indicated in centimeters; dpi = days postinoculation. The insert shows the difference in growth of *Sesbania* plantlets from 1 to 11 days. The distance from the base of the root is marked with the scale. Data are expressed as means $\pm$ standard deviation calculated from three independent experiments. The same letter above the error bars indicates not statistically different according to Duncan's test ($P < 0.05$).

The distribution of root nodules formed by strains ORS571 and AC601.

The inversion in long-term colonization led us to further investigate whether the decrease of the *cheZ* mutant in competitive nodulation results from the decrease in long-term colonization. The distribution of nodules formed by strains ORS571 and AC601 along root surface was characterized, including the number and relative location of nodules. When strains ORS571 and AC601 were inoculated separately, the total number of nodules formed by strains ORS571 and AC601 was similar (Fig. 5A). However, the distribution frequencies of nodules at different positions were significantly different between strains ORS571 and AC601. Most nodules formed by strain AC601 were only found in the root base region, while many nodules of the wild type were located in the root elongation region, toward the root tip (Fig. 5B). These results showed that CheZ contributes to the distribution of nodules.

Then, the distribution of nodules formed by strains ORS571 and AC601 in competitive nodulation assay was determined. When strain ORS571 was mixed with strain AC601 at a 1:1 ratio, the highest numbers of nodules were found at the root base, whereas numbers of nodules gradually decreased in the direction of the root tip (Supplementary Fig. S3). Interestingly, all the nodules formed by strain AC601 were located in the initial susceptible region, and numbers of nodules formed by strain AC601 were less than that formed by strain ORS571 in this region. These results are consistent with the results when strain AC601 and the wild type inoculated separately in long-term colonization, indicating that the *cheZ* mutant could not disperse freely.

EPS may be the key factor contributing to the enhanced early colonization of the *cheZ* mutant.

Similar to the *cheZ* mutant, nonchemotaxis mutants in *Sinorhizobium meliloti* can form normal nodules but had relatively low nodule occupancy in competitive nodulation with the wild type (Caetano-Anollés et al. 1988). In *R. leguminosarum* bv. *trifolii*, production of EPS significantly impaired bacterial attachment and biofilm formation on biotic surfaces (Janczarek et al. 2015). In

Fig. 5. Difference in the distribution of the root nodules formed on *Sesbania* plantlets inoculated by *Azorhizobium caulinodans* wild-type strain ORS571 or by the *cheZ* mutant strain AC601. **A**, Diagrammatic representation of the number of nodules formed with respect to their distance from the root base: between 0 and 2 cm in yellow, 2 and 4 cm in green, and 4 and 6 cm in violet. **B**, Root picture showing nodules localization: the white arrow points to the position of nodules away from the root base. The farthest and youngest nodules in *Sesbania* plantlets by the wild type were magnified. Error bars show $\pm$ standard deviation of the means from at least three independent experiments; ns at the top of the column chart indicate no significant difference between the mutant and wild-type strains according to a χ^2 analysis performed by SPSS.

addition, EPS molecules could promote the formation of infection threads of *R. phaseoli* (Carlson et al. 1987). In order to investigate the role of EPS in the early-term colonization of the *cheZ* mutant, the expression levels of two gene clusters encoding EPS biosynthesis proteins were analyzed (Fig. 6A and B). The relative expression levels of genes in the two clusters all increased more than twofold in the *cheZ* mutant compared with the wild type, especially *oac3* (*azc_1831*). These results indicated that EPS are involved in the CheZ-regulated colonization.

To further investigate the contribution of EPS in the colonization process, a strain carrying a deletion of the *oac3* gene (strain AC1831) and a double mutant strain carrying deletions of both *oac3* and *cheZ* (strain AC6031) were constructed. The Congo red assay and anthrone method were used to assess the

production of EPS produced by these strains qualitatively and quantitatively. EPS production by the *cheZ* mutant (strain AC601) was much higher than that of the wild type, which was in agreement with our previous findings (X. Liu et al. 2018). EPS production by strain AC6031 ($\Delta cheZ \Delta oac3$) was less than that of strain AC601 ($\Delta cheZ$) (Fig. 6C), and strain AC6031 showed a significant decrease in a competitive colonization assay with strain ORS571 (wild type), which was similar to strain AC1831 ($\Delta oac3$) (Fig. 6D), indicating that the increase of EPS production may play important roles in early colonization.

It has been reported that the EPS production is more critical for symbiosis on plant hosts that form indeterminate nodules than on those that form determinate nodules (Mendis et al. 2016). In order to determine whether the type of nodules was affected

Fig. 6. Relative expression of *Azorhizobium caulinodans* *exp* genes in the *cheZ* mutant strain AC601 compared with the wild type. **A**, Schematic representation of the two *exp* clusters in the *A. caulinodans* genome. Cluster I is composed of *azc_1831* (*oac3*), *azc_1832*, *azc_1833*, and *azc_1834*. Cluster II is composed of genes from *azc_3319* to *azc_3332*. **B**, Relative expression of all 17 *exp* genes increased more than fivefold in the *cheZ* mutant compared with the wild type. **C**, Qualitative and quantitative production of exopolysaccharides (EPS) revealed by Congo red staining (top) or by determination in the culture supernatant using the assay described in text (bottom). Strains: ORS571 = wild type, AC601 = *cheZ* mutant, AC1831 = *oac3* mutant, and AC6031 = *cheZ oac3* double mutant. White colonies of AC1831 and AC6031 indicated the lack of EPS production. **D**, Competitive colonization of different strains. Values are means of a standard deviation from at least three independent experiments. Asterisks (*) indicate a significant difference between the wild type and each mutant according to a Student's *t* test.

by CheZ, the number of nodules of different types was counted when inoculated with strain AC601 ($\Delta cheZ$) or strain ORS571 (wild type). However, there was no significant difference in the type of nodules between them (Supplementary Fig. S4).

DISCUSSION

The rhizosphere is a complex environment, and it was established that chemotaxis is important for bacteria to construct effective association with plants (Miller et al. 2007). In *R. solanacearum*, a nonchemotactic mutant could not locate and infect the host plant effectively (Yao and Allen 2006). *B. subtilis* needs chemotaxis to swim toward *Arabidopsis thaliana* roots (Allard-Massicotte et al. 2016). We found that an essential chemotactic gene, *cheZ*, has a negative effect on early-term microbe–plant interaction, which is in contrast to the effect of other chemotactic gene deletions in *Azorhizobium caulinodans* (W. Liu et al. 2018).

When inoculated alone, numbers and nitrogen fixation activities of the nodules induced by the *cheZ* mutant are similar to those of the wild type. Although chemotaxis and motility are not necessary for infection and nodulation, they could confer a significant advantage during these processes (Caetano-Anollés et al. 1988). There is a strong relationship between attachment to the plant root surface and final successful nodulation for rhizobacteria (de Weert et al. 2004). In *R. meliloti* and *Bradyrhizobium japonicum*, motility and chemotaxis significantly increased the initial contact and adsorption of rhizobia to the host root surface, leading to an increase of the efficiency of nodulation (Althabegoiti et al. 2008; Caetano-Anollés et al. 1988). However, when coinoculated with the wild type and the *cheZ* mutant, the nodule occupancy of the *cheZ* mutant was lower than that of the wild type. It is obvious that the competitive advantages of the *cheZ* mutant in early colonization cannot result in the increase of nodule number. Through tracking the dynamics of long-term colonization of the *cheZ* mutant and the wild type separately, we found that the colonization level of the *cheZ* mutant was less than that of the wild type after 5 days. When coinoculated with the wild type, the relative colonization level of the *cheZ* mutant near the root tip decreased faster, indicating that chemotaxis is important in a competitive environment. It is suggested that CheZ plays an essential role in long-term competitive colonization and, once *cheZ* is disrupted, the strains cannot successfully establish effective association with host plants in a complex environment.

In general, nonchemotactic mutants are defective in initial host colonization, yet the *cheZ* mutant strain shows an unusual phenotype in early colonization. What is the reason for the competitive advantage of the *cheZ* mutant? There are two possible explanations implying (i) the alternative cellular function (ACF) (different from motility) associated with the chemotaxis pathway or (ii) the swimming motility in relation to EPS production. (i) For bacteria containing several chemotaxis systems, only one (major) chemotaxis system is involved in the regulation of motility and chemotaxis, while the second or other chemotaxis systems are responsible for ACF such as biofilm formation and EPS production (Black and Yang 2004; Hickman et al. 2005). For other bacteria, chemotaxis and ACF such as adhesion and biofilm can be performed by the same chemotaxis system. For example, the deletion of *cheA* or *cheB* in *Marinobacter adhaerens* resulted in impairment on swimming and biofilm formation at the same time (Sonnenschein et al. 2012). Recently, cross talk was found between the major and minor chemosensory systems on biofilm formation in *Comamonas testosteroni* CNB-1 (Huang et al. 2019). Our group has found that the chemotaxis, flocculation, biofilm formation, and EPS production were regulated by one chemotaxis

system in *A. caulinodans* ORS571, and that the roles of CheA and CheZ antagonize each other during these processes (W. Liu et al. 2018; X. Liu et al. 2018). Deletion of *cheZ* led to improvement of both biofilm formation and EPS production (X. Liu et al. 2018). The inhibition of CheZ on EPS production might be a critical factor for early-term colonization of *A. caulinodans* ORS571. (ii) The altered swimming mode of *cheZ* mutant affects bacterial interactions with surfaces. For many α -Proteobacteria such as *Asticcacaulis biprosthecum*, *Caulobacter crescentus*, and *Agrobacterium tumefaciens*, the contact of cells with a surface could cause an increased synthesis of EPS and promote adhesion to the surface (Belas 2014). The hyperreversal behavior of the *cheZ* mutant may enhance the strength of flagella hitting a solid surface and induce the regulation of mechanosensing on EPS production.

CheZ did not affect nodule formation (number) when inoculated alone; however, the *cheZ* mutant showed a reduced nodule occupancy in a competitive nodulation assay, and the distribution and location of nodules were significantly different between the *cheZ* mutant and the wild type. Nodules can be formed alongside the main root surface of *Sesbania* spp. When the wild type or the *cheZ* mutant was inoculated solely, most nodules were developed in the initial colonizing region of roots. However, nearly no bacterial cells in the *cheZ* mutant can move away from the initial inoculation region (0 to 4 cm). In the competitive nodulation assay, the colonization region of *cheZ* mutant was even narrower (0 to 2 cm), indicating that CheZ is important for the dispersal of nodules along the root. What is the reason for the decreased ability of the *cheZ* mutant to form nodules along young plant roots? (i) The limited areas of colonization and nodulation of the *cheZ* mutant may be consistent with the role of CheZ on swimming ability. Because the wild type displays high efficiency in spreading and utilizing limiting nutrients on the root surface, it is unsurprising that the colonization regions of the *cheZ* mutant become even narrower when coinoculated with the wild type. (ii) EPS, on the one hand, can form a diffusion barrier to protect *Azorhizobium caulinodans* against H_2O_2 (D'Haeze et al. 2004). On the other hand, EPS overproduction might inhibit the migration of the *cheZ* mutant. When we had previously observed the trajectory of the *cheZ* mutant, we found that many cells adhered to the surface of slides, although they were shaking violently and trying to break free (X. Liu et al. 2018). (iii) The *cheZ* mutant is less competitive during infection. At the initial site of colonization, the advantage of the *cheZ* mutant on early colonization can be overturned by the wild type during long-term symbiosis, indicating that nonchemotactic mutants are defective in the maintenance of infection (Terry et al. 2005).

EPS are important for attachment to various surfaces, including root surface (Ma et al. 2006). Overproduction of EPS causes significantly increased adhesion of *Pseudomonas aeruginosa* (Ma et al. 2006), and EPS are also required for the attachment of *Azospirillum brasilense* (Jofré et al. 2004). When the EPS biosynthesis genes are disrupted in *Rhizobium* sp. IRBG74, the resulting mutant strains are defective not only in colonization but also in nodulation (Mittra et al. 2016). In *Azorhizobium caulinodans* ORS571, the roles of EPS in the formation of infection threads and nodulation have been studied (Mittra et al. 2016; Wakao et al. 2015). Consistent with previous reports (Gao et al. 2001; Goethals et al. 1994), deletion of *oac3* abolished the EPS production in *A. caulinodans* ORS571. In the present work, a mutant strain carrying a deletion of both *oac3* and *cheZ* (strain AC6031) not only resulted in a decrease of EPS production but also showed reduced early colonization, a phenotype which is similar to that of the *oac3* mutant strain (AC1801) but different from the *cheZ* mutant (strain AC601), indicating that the enhanced early-term colonization of the *cheZ* mutant might depend on the increase of EPS production.

In addition, the enhanced expression of *eps* synthesis genes in the *cheZ* mutant further indicated that EPS may be the key factor in *cheZ* mutant early- and long-term colonization.

As quoted before, multiple chemosensory systems in bacteria control ACF. They are involved in the control of EPS production (Black and Yang 2004), biofilm formation (Huangyutitham et al. 2013), motility by type IV pili (Bhaya et al. 2001), flagella biosynthesis (Berleman and Bauer 2005a), fruiting-body formation (Willett and Kirby 2011), developmental gene expression regulation (Kirby and Zusman 2003), cyst formation (Berleman and Bauer 2005b), and swarm cell differentiation (Burkart et al. 1998). For example, in *Myxococcus xanthus*, the EPS production is regulated by the Dif chemosensory system, which could not regulate motility (Black et al. 2015). Recently, in *C. crescentus*, chemotactic protein CheB, encoded by a *cheB* gene that is not part of the major chemotaxis operon, was found to play a role in surface attachment, antagonizing the role of CheA (Berne and Brun 2019). Although CheZ in *A. caulinodans* also functions in an antagonistic manner with CheA, it could regulate EPS production and chemotactic motility at the same time.

It has been reported that flagella-mediated chemotaxis enables bacteria to reach suitable niches but is not essential for the subsequent contacts of bacteria with the host plants (Coskun et al. 2017). In this work, we found that CheZ cannot only regulate early attachment via affecting EPS production but also influences the dispersal of bacteria along root surface in long-term colonization. The effect of chemotactic proteins in plant-microbe relationships at the colonization level could enable us to use microbes to enhance agriculture productivity effectively.

MATERIALS AND METHODS

Media and growth conditions.

The bacterial strains and plasmids used in this study are listed in Table 1. The *A. caulinodans* strains (Dreyfus et al. 1988), including the wild type and its derivatives, were cultured at 37°C in tryptone-yeast extract (TY) medium overnight. *A. caulinodans* strains were added with antibiotics at the following final concentrations: ampicillin at 100 µg/ml; nalidixic acid at 25 µg/ml; gentamicin at 50 µg/ml; kanamycin at 50 µg/ml; and tetracycline at 100 µg/ml.

Plasmid and strain construction.

To construct the *oac3* mutant, a 626-bp upstream fragment and a 751-bp downstream fragment of *oac3* were amplified by PCR. The amplicons of the upstream fragment and allelic exchange vector pCM351 (Marx and Lidstrom 2002) were digested with restriction enzymes (*KpnI* and *NdeI*); then, they were linked with T4 ligase. The resulting construct and

downstream fragment were digested with restriction enzymes (*AgeI* and *SacI*) and linked by T4 ligase. The modified pCM351 containing the upstream and downstream fragments of *oac3* was introduced into strain ORS571 by triparental conjugation for allelic exchange with the helper pRK2013, as previously described (Ditta et al. 1980). Homologous recombinants carrying a deletion of the *oac3* gene were selected on TY plates containing gentamicin, nalidixic acid, and ampicillin. It was checked that the recombinant strain cannot grow on plates containing tetracycline. Double recombination was confirmed by PCR and sequencing. The selected strain, named AC1831, was used in subsequent experiments.

The *cheZ* mutant strain AC601 was used to obtain a double-mutant strain carrying a deletion of *oac3* and *cheZ* genes. First, the gentamicin gene was removed using pCM157, as previously described (Marx and Lidstrom 2002). Then, the resulting unmarked *cheZ* mutant was used to construct a double-deletion mutant ($\Delta cheZ \Delta oac3$) and the selected strain, named AC6031, was used in subsequent experiments.

Plant growth.

Seed sterilization and germination were performed as previously described (Jiang et al. 2016). Seedlings of similar root size were selected and used in subsequent experiments.

Early-term colonization assay.

For early-term colonization, strains ORS571 and AC601 were mixed with equal ratios and plants were soaked in the cell suspension. Incubation was performed with shaking at 50 rpm, at the time course indicated in the Results section. The bacteria tightly attached to the roots were collected and quantified. Adsorbed rhizobia were collected from the root surfaces, and the recovered isolates from surfaces were identified by PCR (Mongiardini et al. 2008). During the time-course assay, *Sesbania* roots were inoculated from 10 to 240 min.

Long-term colonization assay.

Seedlings were soaked in bacterial suspensions using *A. caulinodans* strains marked with *lacZ*. The concentration of bacteria was adjusted to an OD₆₀₀ of 0.6. Then, roots were stained with 5-bromo-4-chloro-3-indolyl-beta-D-galacto-pyranoside and the colonization patterns at different times were analyzed, as previously reported (Qiu et al. 2015). For competitive long-term colonization assay, strains ORS571 and AC601 were mixed at equal proportions prior to inoculation. After growing for different periods of time, roots were washed at least five times, and the different regions of roots were collected separately. The roots were vortexed and bacteria were reisolated on TY solid agar plates containing nalidixic acid and ampicillin. The proportion between strains ORS571 and AC601 was quantified by TY solid plates containing gentamicin, where strain ORS571 could not survive.

Table 1. Bacteria strains and plasmids used in this study

Strain or plasmid	Relevant characteristics ^a	Source or reference
Strains		
<i>Escherichia coli</i>		
DH5α	F ⁻ supE44 AlacU169 (φ80 lacZΔM15) hsdR17 recA1 endA1 gyrA96 thi-1 relA1	Transgen
<i>Azorhizobium caulinodans</i>		
ORS571	Type strain; Am ^R , Na ^R	Dreyfus et al. 1988
AC601	ORS571 derivative; Δ <i>cheZ</i> , Am ^R , Na ^R , Gm ^R	X. Liu et al. 2018
AC1831	ORS571 derivative, Δ <i>oac3</i> , Am ^R , Na ^R , Gm ^R	This study
AC6031	AC601 derivative, Δ <i>cheZ</i> and Δ <i>oac3</i> , Am ^R , Na ^R , Gm ^R	This study
Plasmids		
pCM351	Allelic exchange vector, Gm ^R , Tc ^R	Marx and Lidstrom 2002
pCM157	Allelic exchange vector, Tc ^R	Marx and Lidstrom 2002
pRK2013	Helper plasmid, ColE1 replicon; Tr ^R , Km ^R	Ditta et al. 1980

^a Am^R, Na^R, Gm^R, Tc^R, and Km^R = resistant to ampicillin, nalidixic acid, gentamicin, tetracycline, and kanamycin, respectively.

Nodulation of *Sesbania rostrata*.

For root nodulation assays, surface-sterilized seedlings that were similar in size and length were selected and inoculated with *A. caulinodans* strains adjusted to an OD₆₀₀ of 0.6 for 4 h. Then, seedlings were transplanted into Leonard jars filled with sterilized vermiculite. The nodulation pattern was analyzed after approximately 45 days. For stem nodulation assays, after *Sesbania rostrata* grew for 4 weeks, *A. caulinodans* strains adjusted to an OD₆₀₀ of 0.6 were uniformly painted alongside the stem surface. The stem nodulation pattern was analyzed after another 4 weeks.

Expression analysis of EPS biosynthesis-related genes.

Strains ORS571 and AC601 were cultured in TY liquid medium at 37°C for 24 h. TransZol Up Plus RNA kit (Transgen Biotech) was used to isolate total RNA from 25 ml of *A. caulinodans* cells. After determining the concentration and quality of RNA via Nanodrop 2000, a One-Step gDNA removal kit (Transgen Biotech) was used to synthesize cDNA for subsequent experiments. GoTaq qPCR Master Mix kit (Promega Corp.) was used to perform real-time quantitative PCR with the gene-specific primer pairs listed in Supplementary Table S1. The transcript levels of 16sRNA were used as a control, and the comparative cycle threshold method (Schmittgen and Livak 2008) was used to analyze the transcript levels of *exp* genes.

Qualitative and quantitative assays of EPS production.

The qualitative assay of EPS production was performed as previously described (Nakajima et al. 2012), with some modifications, as described by Jiang et al. (2016). Qualitative determination using Congo red staining was as described by Jiang et al. (2016). The quantitative assay of EPS production was performed using the anthrone method with some modifications, as described by X. Liu et al. (2018). These experiments were repeated at least three times, with six replicates per sample.

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