Protein Residues and a Novel Motif Involved in the Cellular Localization of CheZ in *Azorhizobium caulinodans* ORS571

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Chemotaxis is essential for the competitiveness of motile bacteria in complex and harsh environments. The localization of chemotactic proteins in the cell is critical for coordinating a maximal response to chemotactic signals. One chemotaxis protein with a well-defined subcellular localization is the phosphatase CheZ. CheZ localizes to cell poles by binding with CheA in *Escherichia coli* and other enteric bacteria, or binding with a poorly understood protein called ChePep in epsilon-Proteobacteria. In alpha-Proteobacteria, CheZ lacks CheA-binding sites, and its cellular localization remains unknown. We therefore determined the localization of CheZ in the alpha-Proteobacteria *Azorhizobium caulinodans* ORS571. *A. caulinodans* CheZ, also termed as CheZ_{AC}, was found to be located to cell poles independently of CheA, and we suspect that either the N-terminal helix or the four-helix bundle of CheZ_{AC} is sufficient to locate to cell poles. We also found a novel motif, AXXFQ, which is adjacent to the phosphatase active motif DXXXQ, which effects the monopolar localization of CheZ_{AC}. This novel motif consisting of AXXFQ is conserved in CheZ and widely distributed among Proteobacteria. Finally, we found that the substitution of phosphatase active site affects the polar localization of CheZ_{AC}. In total, this work characterized the localization pattern of CheZ containing a novel motif, and we mapped the regions of CheZ_{AC} that are critical for its polar localization.

**Keywords:** chemotaxis, CheZ, cellular localization, *Azorhizobium caulinodans*, rhizobia

**INTRODUCTION**

In harsh and complex environments, bacteria must adapt and respond to external changes quickly. Chemotaxis systems are one-way bacteria have evolved to do this. Chemotaxis enables bacteria to regulate their motility in response to environmental signals. The chemotaxis pathway has been well studied in *Escherichia coli*. External signals or nutrient molecules are sensed by chemoreceptors. Upon binding with attractant signals, conformational changes of chemoreceptors inhibit the autokinase activity of the associated histidine kinase CheA. In the presents of a repellent signal,
CheA can phosphorylate the response regulator CheY, and CheY-P diffuses and binds with the flagellar motor proteins FlhM and Fi1N, causing the flagella to change rotational direction from counterclockwise to clockwise (Szurmant and Ordal, 2004). The phosphatase CheZ promotes the intrinsic dephosphorylation of CheY-P to terminate the signal transduction (Blat and Eisenbach, 1994; Silversmith et al., 2008; Silversmith, 2010).

The spatial organization of chemotaxis proteins is critical for bacterial chemotaxis to adapt to environments. Chemotaxis proteins are localized to cellular poles using multiple strategies, including the nucleoid occlusion, Tol/Pal complex, membrane curvature, and protein-protein interactions (Laloux and Jacobs-Wagner, 2014). Transmembrane *E. coli* chemoreceptors maintain polar localization through the Tol/Pal complex, strong membrane curvature, or nucleoid exclusion (Santos et al., 2014; Neeli-Venkata et al., 2016; Saaki et al., 2018). The Tol/Pal complex is a conserved component of bacterial cell envelope, which is involved in the maintenance of cell wall integrity (Bernadac et al., 1998). Other chemotaxis proteins including CheA, CheW, CheY, and CheZ locate to cellular poles based on the interaction with other chemotaxis proteins (Sourjik and Berg, 2000). CheA and CheW can bind to chemoreceptor forming polar chemotaxis complexes (Pinas et al., 2016), and the localization of CheZ and CheY depends on the presence of CheA in *E. coli* (Sourjik and Berg, 2000).

CheZ is encoded in around 40% of bacterial genomes (Wuichet and Zhulin, 2010), and the localization mechanism of CheZ has been well studied in *E. coli*. *E. coli* CheZ, termed as CheZEC, locates to cellular poles with the help of CheA-short (CheAs), a short form of CheA lacking the first 97 amino residues of full length CheA, called CheA-long. CheZEC interacts with CheA using a small region of amino acids with most interactions coming from the apical hairpin loop consisting of two aromatic residues, Phe-97 and Trp-98 (Cantwell and Manson, 2009). For CheAs, two hydrophobic residues Leu-123 and Leu-126 in the N-terminus of CheA are responsible for CheZEC interactions (Cantwell et al., 2003; Hao et al., 2009).

*Azoarcobacterium caulinothans* ORS571 is a rhizobium belonging to alpha-Proteobacteria uses chemotaxis for plant colonization. It fixes nitrogen with the host *Sesbania rostrata* by forming stem or root nodules (Dreyfus et al., 1983). *A. caulinothans* ORS571 has only one chemotaxis pathway including one gene cluster (cheA, cheW, cheY2, cheB, and cheR) and two orphan genes (cheY1 and cheZ) (Jiang et al., 2016). Deletion of one or several genes within the *A. caulinothans* ORS571 chemotaxis cluster reduces or abolishes the chemotaxis of *A. caulinothans* ORS571, confirming the role of these genes in chemotaxis (Liu W. et al., 2018; Liu X. et al., 2018). Deletion of cheZ causes *A. caulinothans* non-chemotactic, while in contrast to other chemotaxis proteins which are important for host plant colonization, CheZ plays negative roles on early colonization (Liu et al., 2019). We previously found that a soluble heme-binding chemotaxis protein in *A. caulinothans* locates at the cell poles with the help of CheA (Jiang et al., 2016). However, it has been reported that CheZ proteins in alpha- and delta-Proteobacteria lack the sequences responsible for CheAs binding (Wuichet et al., 2007), and the localization of CheZ in alpha-Proteobacteria remains unknown.

In the present study, we reported the localization pattern of CheZ in *A. caulinothans* ORS571 and mapped regions of CheZAC that are sufficient for polar localization by constructing various truncated mutants of CheZAC. Furthermore, a novel motif in CheZAC, which is conserved among Proteobacteria, was found to be involved in the regulation of monopolar CheZ localization.

**RESULTS**

**Bioinformatics Analysis Shows that CheZAC Lacks Canonical Sites Involved in CheZEC Polar Localization**

The structure of CheZEC consists of four regions, including an N-terminal helix (residues 1–34) of unknown functions, a four-helix bundle formed from a dimer of two hairpin structures (residues 35–168), a linker (residues 169–199), and a C-terminal helix (residues 199–214) (Zhao et al., 2002; Silversmith, 2005). Because the amino acid sites involved in the CheZEC polar localization were well studied, we first aligned the CheZ amino acid sequences from *E. coli* (CheZEC) and *A. caulinothans* (CheZAC) using an EMBASS Needle program (Madeira et al., 2019). Then, we modeled the structure of CheZAC using online server SWISS-MODEL (Waterhouse et al., 2018) and Ipred4 (Drozdzetskiy et al., 2015).

An alignment of CheZAC and CheZEC proteins showed significant similarity (33.9%) between them and both of them have conserved phosphatase active sites (Asp 165 and Gln 169 in CheZAC) (Figure 1A), which is consistent with our previous report (Liu X. et al., 2018). Structure modeling results (Figures 1A,B) suggested CheZAC also has the N-terminal helix (residues 1–86 in CheZAC), four-helix bundle hairpin (residues 87–195 in CheZAC), the linker (residues 196–225 in CheZAC), and the C-terminal helix (residues 225–236 in CheZAC). Interestingly, the N-terminal helix in CheZAC is substantially longer (>50 residues) than CheZEC, while the four-helix bundle hairpin (>25 residues) in CheZAC is substantially shorter than CheZEC (Figure 1). And, the gaps in the alignment are similar in size on either side of the hairpin turn (residues ~140), which is consistent with a shorter bundle (Figures 1A,B). Remaining of residues from 70 to 133 including the tip of hairpin (residues ~100) is sufficient for polar localization of CheZEC (Cantwell and Manson, 2009). The similarity of the hairpin tip between CheZAC and CheZEC indicates that the hairpin tip might be also employed by CheZAC to bind potential localization partner proteins. However, a conserved motif D(D/E)WF (residues 95–105) was found to be important for CheZEC polar localization, which was not found in CheZAC (Figure 1A).

In *E. coli*, the polar localization of CheZ is achieved by binding with a short form of CheA (CheAEC), which begins at Met-98 of full length CheAEC (Cantwell et al., 2003; Hao et al., 2009). There is only one CheA protein encoded in *A. caulinothans* genome, termed as CheAEC. When we made a pairwise sequence alignment of CheAEC and CheAEC, the absence of cognate CheAEC Met-98 in CheAEC suggests that CheAEC does not have a short form of CheA (Supplementary Figure S1). These results
suggest that CheZ<sub>AC</sub> may not locate to cell poles or locate to cell poles with a different mechanism from CheZ<sub>EC</sub>.

**Characteristics of CheZ Localization in A. caulinodans ORS571**

To study the subcellular localization of CheZ in A. caulinodans cells, we designed a C-terminal GFP fusion to CheZ<sub>AC</sub>. To avoid artifacts related overexpression, the expression of the fusion gene was controlled by the native promotor of cheZ (Liu X. et al., 2018). When the CheZ<sub>AC</sub> fusion was expressed in the cheZ mutant strain, the chemotactic behavior of the cheZ null mutant was partially complemented to wild-type levels that contains a control vector pBBR2GFP, indicating the CheZ-GFP retains function (Figure 2A). Another evidence showing that CheZ-GFP functions is that the presence of CheZ-GFP restores ΔcheZ flagella rotating to wild-type level. Wild type and ΔcheZ complemental strains both rotate their flagella between clockwise and counter-clockwise, while ΔcheZ with or without pBBR2GFP always shows counter-clockwise rotation (Unpublished data).

We next determined the spatial distribution of CheZ-GFP by fluorescence microscopy. The CheZ-GFP fusion showed three unique localization patterns (Figure 2B). We manually determined and quantified these localization patterns using ImageJ, comparing the brightness of polar foci with that of cell body (see Materials and Methods). About 60% of cells demonstrated diffuse CheZ-GFP localization, 30% of cells CheZ-GFP localized to both cell poles, and 10% of cells showed monopolar localization (Figure 3B). The percent of cells with polar localized CheZ in A. caulinodans are significantly lower than that in E. coli, in which CheZ<sub>EC</sub> shows polar localization in 85% of the cells (Blat and Eisenbach, 1996). Except polar and diffuse localized pattern, there were also many lateral clusters of CheZ-GFP (Figure 2C). When analyzing the distance from each lateral cluster to the pole of cell, the position of each lateral cluster distributed along the cell body with a period corresponding to the 1/2 or 1/4 of the cell length (Figure 2C).

**The Polar Localization of CheZ<sub>AC</sub> Is Independent of Chemotaxis and Flagellar Proteins**

Numerous studies have shown that chemotaxis protein can form a polar cluster to better adapt to environmental conditions. To study whether the polar localization of CheZ<sub>AC</sub> is dependent on CheA or other chemotaxis proteins, we examined the localization patterns of CheZ<sub>AC</sub> in different backgrounds lacking different chemotaxis proteins. Consistent with our bioinformatics analysis, deletion of cheA does not alter the cellular localization of CheZ<sub>AC</sub> (Figure 3). We then tested the localization pattern of CheZ<sub>AC</sub> in the following chemotaxis mutants, ΔcheY1, ΔcheY2, or ΔcheA-R clusters (including cheA, cheY2, cheW, cheB, and cheR) (Liu W. et al., 2018; Liu et al., 2020). Interestingly, CheZ<sub>AC</sub> maintains polar localization in both the ΔcheY1, ΔcheY2, and ΔcheA-R mutants backgrounds (Figure 3).

Next, we tested if flagellar proteins affect CheZ<sub>AC</sub> polar localization. FliM and FliN are two flagellar motor components and interact with CheY either directly or indirectly (Delalez et al., 2014). Deletion of either one makes A. caulinodans non-flagellated and non-motile (Shen et al., 2018), however, neither of them abolishes the polar localization of CheZ<sub>AC</sub> (Figure 3).
These results indicate that CheZ\textsubscript{AC} is localized to the cell poles independent of chemotaxis or flagellar proteins.

**N-terminal Helical Regions Are Sufficient for CheZ\textsubscript{AC} Polar Localization**

Although there is low conservation between *E. coli* and *A. caulinodans* CheZ, the C-terminal sequences including CheY-P binding region and phosphatase active sites are conserved (Blat and Eisenbach, 1996; Zhao et al., 2002; Wuichet et al., 2007; Silversmith, 2010; Liu X. et al., 2018). The N-terminal helix, whose function remains unknown, and the middle four-helix bundle hairpin of CheZ, which is required for localization in *E. coli*, are variable (Lertsethtakarn and Ottemann, 2010). To map the region sufficient for polar localization, various portions of the N-terminal helix (residues 1–86), and middle four-helix bundle regions (residues 87–195) of CheZ\textsubscript{AC} were fused to GFP (Figure 4A). CheZ\textsubscript{Δ51-236}, containing a portion of N-terminal helix of CheZ\textsubscript{AC}, failed to localize to the cell poles (Figures 4B,C). Surprisingly, CheZ\textsubscript{Δ1-236}, containing nearly all regions of the N-terminal helix can locate to cellular poles, though the number of cells with bipolar localized CheZ\textsubscript{AC} decreased from 30 to 5% compared to full-length CheZ\textsubscript{AC} (Figures 4B,C). Because the four-helix bundle, especially its hairpin tip, is essential for the polar localized pattern of CheZ\textsubscript{EC} (Hao et al., 2009), we made a longer truncated mutant containing a portion of the four-helix bundle CheZ\textsubscript{AC140-236}. Intriguingly, CheZ\textsubscript{AC140-236} can localize to cell poles, however, the cell ratio showed polar localized pattern decreased no more than 5%. When the remaining residues extended from 1–139 to 1–169, including almost all the region of N-terminal helix and four-helix bundle, the CheZ\textsubscript{AC170-236} mutant can locate to mono- or bi-polar poles in cells above 70%
A. caulinodans for the polar localization in bundle is involved in the regulation of CheZ polar localization. AC sufficient for polar localization of CheZ different from that of CheZ EC. The N-terminal helical region is sufficient for polar localization of CheZ AC, and the four-helix bundle is involved in the regulation of CheZ polar localization.

To further determine the regions of CheZ that are responsible for the polar localization in A. caulinodans, CheZ AC proteins with various deletions at the N-terminal helix and four-helix bundle were fused to GFP (Figure 4A). All the fusion proteins were introduced into the cheZ mutant and expressed with the native promoter. When part of the CheZ AC N-terminal helix was deleted, including residues from 2 to 31 or from 2 to 50 (termed as CheZΔ2-31 and CheZΔ2-50), the truncated mutant maintained polar localization, though the polar localized CheZ AC decreased from 40 to 25, and 10%, respectively (Figures 4B,C). Unexpectedly, deletions of N-terminal helical regions from residues 2–70 (CheZACΔ2-70) or 51–70 (CheZACΔ51-70) did not abolish the polar localization of CheZ AC (Figures 4B,C). These results suggest that the region from residues 2–70 might not be the sole region sufficient for polar localization of CheZ AC. We further tested the polar localized pattern of mutants lacking part of the four-helix bundle hairpin, CheZΔ71-100, CheZΔ97-137, and CheZΔ138-169, and we found that all of them remained the polar location of CheZ AC. These results suggest that CheZ AC might be anchored to cell poles via multiple motifs. Interestingly, CheZΔ138-169 not only maintains polar localization, but also shows 100% monopolar localized pattern (Figure 4).

**Mining for a Novel Motif Involved in the Regulation of CheZ AC Localization**

We next sought to identify what protein or residues are responsible for the unique monopolar localization pattern of CheZ Δ138-169. When CheZ Δ138-169 was introduced into various chemotaxis and flagella mutants, it always maintained nearly 100% monopolar localization (Supplementary Figure S2). These results suggest that the role of residues from 138 to 169 on the localization of CheZ AC is not affected by other chemotaxis or flagellar proteins.

To find potential conserved sites required for the monopolar localized pattern of CheZ, we analyzed twenty-five amino acid sequences of CheZ proteins from alpha-Proteobacteria which are closely related to A. caulinodans (Figure 5A). Within residues 138–169, two conserved features were found. One is the conserved phosphatase motif (DXXXQ) (Lertsethtakarn and Ottemann, 2010), and the other is an uncharacterized conserved motif (ACNFQ), which is close to the phosphatase motif (Figures 5B,C). Interestingly, deletion of the ACNFQ motif (CheZ Δ158-164) was sufficient to cause the monopolar localized pattern of CheZ AC (Figure 5D). To further investigate whether the monopolar localization resulted from the deletion of ACNFQ, three point-directed mutants, A160R, C161A, and F163L, were constructed successfully. The localization pattern of CheZ_C161A and CheZ_F163L were similar with that of wild-type CheZ AC, indicating these residues are not required. CheZ_A160R showed different localization that was nearly 100% bipolar (Figure 5E). These results suggest that the novel motif ACNFQ is involved in the monopolar localization of CheZ AC and conserved site A160 within the motif might contribute more to the function.

**The AXXFQ Motif Is Conserved in Proteobacteria**

CheZ distributes broadly among alpha-, beta-, gamma-, delta-, and epsilon-Proteobacteria (Wuichet et al., 2007). Although the degree of identity and similarity between these CheZ proteins are low, the catalytic residues in phosphatase active motif are highly conserved.
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Conserved among them (Wuichet et al., 2007). To determine the distribution of the novel motif ACNFQ in Proteobacteria, the representative CheZ sequences from each class (alpha-, beta-, gamma-, delta-, and epsilon-Proteobacteria) were selected for alignment. All these CheZ proteins have the novel ACNFQ motif close to phosphatase sites (Figure 6A), although the second and third amino acids in the motif are variable among Proteobacteria, which was renamed as AXXFQ motif. We then used more than 200 representative sequences from each class to align and build a WebLogo of the conserved region consensus sequences (Crooks et al., 2004). The glutamine residue (Q164 in A. caulinodans) near phosphatase sites DXXXQ is the most conserved site among different Proteobacteria (Figure 6B). Alanine and phenylalanine residues (A160 and F163 in A. caulinodans) are the second conserved sites (Figure 6B). In epsilon-Proteobacteria, there is a tyrosine residue instead of phenylalanine (Figure 6B). Considering both tyrosine and phenylalanine have a benzene ring, this might be a conservative substitution. These results showed that the novel motif AXXF(Y)Q is widely distributed and conserved among Proteobacteria.

Phosphatase Active Sites Affect CheZ<sub>AC</sub> Location

The proximity between these two motifs (AXXFQ and DXXXQ) led us to assess if the localization pattern could be affected by phosphatase active sites. To investigate the role of phosphatase activity on the subcellular localization of CheZ<sub>AC</sub>, site-directed mutants of D165A and Q169A, both critical for the phosphatase activity of CheZ<sub>AC</sub> (Zhao et al., 2002), were fused to GFP. CheZ_Q169A showed a small increase in diffuse localization, and D165A caused an obvious decrease of polar localization (Figures 6C,D), suggesting the subcellular localization of CheZ<sub>AC</sub> might be affected by phosphatase active sites. Because the localization of CheZ<sub>AC</sub> is affected by phosphatase active sites, in turn, the role of different regions
affecting localization on chemotactic behavior were assessed. Five truncated mutants, CheZAΔ2-31, CheZAΔ2-50, CheZAΔ2-70, CheZAΔ51-70, and CheZAΔ71-100, restored the chemotaxis of cheZ mutant (Figure 4A and Supplementary Figure S3), suggesting that the N-terminus of CheZA is not essential for its phosphatase activity.

DISCUSSION

The location of CheZ to cell poles can improve the sensitivity to chemotactic stimuli. In E. coli, CheZΔF98S, a CheZEC variant that abolished localization, showed a decreased chemotactic response to external signals (Cantwell et al., 2003; Vaknin and Berg, 2004). Furthermore, the spatial distribution of chemotactic proteins, including CheZ, provides a region for specialized functions which are similar as the membrane-bound organelles in eukaryotic cells (Maddock and Shapiro, 1993). Three localization patterns of CheZ were found in A. caulinodans, diffuse, bipolar, and monopolar, indicating the localization pattern of CheZ in A. caulinodans is more complex than that in E. coli (Sourjik and Berg, 2000; Cantwell et al., 2003). The ratio of cells that demonstrated polar localization of CheZA is much lower than that of CheZEC, indicating the role of CheZA localization may be different between them. Similar to CheZ in E. coli, the localization of CheZ at lateral body of A. caulinodans cells showed a typical periodic distribution, and this phenomenon may be interpreted by a “stochastic nucleation model” (Greenfield et al., 2009; Saaki et al., 2018).

For many bacteria, CheZ locates near cell poles where CheY-P is generated, and the phosphatase activity of CheZ is 5- to 10-folds higher at the position (Vaknin and Berg, 2004). The enhanced phosphatase activity of CheZ ensures that peritrichously located flagellar motors experience a uniform concentration of CheY-P, which is critical for the coordinated regulation of flagellar motility (Cluzel et al., 2000; Lipkow et al., 2005; Ringgaard et al., 2011). We determined CheZA still locate to cellular poles despite lacking a CheA binding site (Wuchet et al., 2007) or other chemotaxis or flagellar proteins. The localization of a remote CheZ ortholog in Helicobacter pylori has been studied (Lertsethtakarn and Ottemann, 2010; Lertsethtakarn et al., 2015). And, in contrast to E. coli CheZ, H. pylori CheZ localization is
TABLE 1 | Bacteria strains and plasmids used in this study.

| Strain or plasmid | Relevant characteristics<sup>a</sup> | Source or references |
|-------------------|--------------------------------------|----------------------|
| **Strains**<sup>b</sup> | | |
| E. coli DH5α | F- supE44 lacU169 (ΔrpoA ΔlacI ΔM15) hsdR17 recA1 endA1 gyrA96 thi-1 relA1 | Transgen |
| Azorhizobium caulinodans | | |
| cheZ mutant | ORS571 derivative; ΔcheZ, Amp<sup>R</sup>, Nal<sup>R</sup>, Gm<sup>R</sup> | Liu X. et al., 2018 |
| cheZ mutant derivatives | ΔcheZ completed with different CheZ variants, Amp<sup>R</sup>, Nal<sup>R</sup>, Gm<sup>R</sup>, Km<sup>R</sup> | This study |
| **Plasmids** | | |
| pRK2013 | Helper plasmid, ColE1 replicon; Tra + Km<sup>R</sup> | Ditta et al., 1980 |
| pBBR1MCS-2 | Broad host range plasmid, Km<sup>R</sup> | Kovach et al., 1995 |
| pBBRCheZ | pBBR-1-MCS-2 with cheZ open reading frame and 406-bp upstream promoter region; Km<sup>R</sup> | This study |
| pBBRGFP | pBBR-1-MCS-2 with egfp gene; Km<sup>R</sup> | This study |
| pBBRCheZD165A | pBBR-1-MCS-2 with cheZ site substitution mutant and 406-bp upstream promoter region; Km<sup>R</sup> | Liu X. et al., 2018 |
| pBBRCheZQ169A | pBBR-1-MCS-2 with cheZ site substitution mutant and 406-bp upstream promoter region; Km<sup>R</sup> | Liu X. et al., 2018 |
| pBBRCheZ-GFP | pBBR-1-MCS-2 with cheZ fused with egfp; Km<sup>R</sup> | This study |
| pBBRCheZΔ2-31-GFP | pBBR-1-MCS-2 with cheZ lacking bases from 4–94 bp fused with egfp; Km<sup>R</sup> | This study |
| pBBRCheZΔ2-50-GFP | pBBR-1-MCS-2 with cheZ lacking bases from 4–150 bp fused with egfp; Km<sup>R</sup> | This study |
| pBBRCheZΔ2-70-GFP | pBBR-1-MCS-2 with cheZ lacking bases from 4–210 bp fused with egfp; Km<sup>R</sup> | This study |
| pBBRCheZΔ51-70-GFP | pBBR-1-MCS-2 with cheZ lacking bases from 151–210 bp fused with egfp; Km<sup>R</sup> | This study |
| pBBRCheZΔ71-100-GFP | pBBR-1-MCS-2 with cheZ lacking bases from 211–300 bp fused with egfp; Km<sup>R</sup> | This study |
| pBBRCheZΔ97-137-GFP | pBBR-1-MCS-2 with cheZ lacking bases from 292–411 bp fused with egfp; Km<sup>R</sup> | This study |
| pBBRCheZΔ138-169-GFP | pBBR-1-MCS-2 with cheZ lacking bases from 412–507 bp fused with egfp; Km<sup>R</sup> | This study |
| pBBRCheZΔ71-236-GFP | pBBR-1-MCS-2 with cheZ lacking bases from 211–708 bp fused with egfp; Km<sup>R</sup> | This study |
| pBBRCheZΔ51-236-GFP | pBBR-1-MCS-2 with cheZ lacking bases from 151–708 bp fused with egfp; Km<sup>R</sup> | This study |
| pBBRCheZΔ140-236-GFP | pBBR-1-MCS-2 with cheZ lacking bases from 418–708 bp fused with egfp; Km<sup>R</sup> | This study |
| pBBRCheZΔ170-236-GFP | pBBR-1-MCS-2 with cheZ lacking bases from 508–708 bp fused with egfp; Km<sup>R</sup> | This study |
| pBBRCheZΔ158-164-GFP | pBBR-1-MCS-2 with cheZ lacking bases from 472–492 bp fused with egfp; Km<sup>R</sup> | This study |
| pBBRCheZC161A-GFP | pBBR-1-MCS-2 with cheZ with a C161A substitution fused with egfp; Km<sup>R</sup> | This study |
| pBBRCheZF163L-GFP | pBBR-1-MCS-2 with cheZ with a F163L substitution fused with egfp; Km<sup>R</sup> | This study |
| pBBRCheZA160R-GFP | pBBR-1-MCS-2 with cheZ with an A160R substitution fused with egfp; Km<sup>R</sup> | This study |
| pBBRCheZD165A-GFP | pBBR-1-MCS-2 with cheZ with a C161A substitution fused with egfp; Km<sup>R</sup> | This study |
| pBBRCheZQ169A-GFP | pBBR-1-MCS-2 with cheZ with a C161A substitution fused with egfp; Km<sup>R</sup> | This study |

<sup>a</sup>Amp<sup>R</sup>, ampicillin resistance; Gm<sup>R</sup>, gentamicin resistance; Km<sup>R</sup>, kanamycin resistance; Nal<sup>R</sup>, nalidixic acid; Te<sup>R</sup>, tetracycline.

independent of CheA or other typical chemotaxis proteins, but dependent on ChePep, a novel chemotaxis protein distributed among epsilon-<i>Proteobacteria</i> (Howitt et al., 2011; Lertsethtakarn et al., 2015). Because the genes encoding homologs of ChePep were not found in <i>A. caulinodans</i> genome, we suspect that there might be other partner proteins that contribute CheZ to localization clusters.

The four-helix bundle of CheZ<sub>EC</sub>, especially the tip of the hairpin, is responsible for polar localization in <i>E. coli</i>. In this work, we found that the N-terminal helix is sufficient for the polar localization of CheZ<sub>AC</sub>. The sequence conservation of N-terminus of CheZ between <i>E. coli</i> and <i>A. caulinodans</i> is low, and interestingly, deletion of the N-terminal helix, CheZ<sub>AC</sub> still remained the polar location, indicating more than one region is sufficient for its polar localization. However, the reason why CheZ<sub>AC</sub> has two independent regions that are sufficient for polar localization is unknown.

CheZ<sub>Δ138-169</sub> results in monopolar localization. In this study, a conserved motif AXXF(Y)Q which is close to the phosphatase active motif DXXXQ was found to be responsible for the unique monopolar localized pattern of CheZ<sub>AC</sub>. Although AXXF(Y)Q is conserved among <i>Proteobacteria</i>, its role in localization and chemotaxis had not been studied. We speculate the high level of polar localization of CheZ in <i>E. coli</i> and <i>H. pylori</i> under common conditions may mask the observation of the role of AXXF(Y)Q on localization changes (Sourjik and Berg, 2000; Cantwell et al., 2003; Lertsethtakarn et al., 2015). The biological significance of the polar localization is that each daughter cell can inherit a CheZ after cell division (Jones and Armitage, 2015; Mauriello et al., 2018). For example, the location of chemotactic proteins transfers from monopolar to bipolar clusters in <i>Vibrio cholerae</i> before cell division (Ringgaard et al., 2011). The unipolar localization of CheZ<sub>Δ138-169</sub> or CheZ<sub>Δ158-164</sub> indicates that one daughter cell cannot inherit CheZ<sub>AC</sub>, and the residues 138–169 might be involved in the dissociation between CheZ<sub>AC</sub> and its binding partners for localization. In <i>E. coli</i>, the polar localization of CheZ<sub>AC</sub> can be improved by the interaction with CheA at the chemotaxis signaling complex (Wang and Matsumura, 1996;
Vaknin and Berg, 2004). These results further suggest that there may be other proteins that recruit CheZ to the clusters and/or affect the catalysis activity of CheZ, as seen in H. pylori (Lertsethtakarn et al., 2015). In this study, we mapped the critical regions sufficient for CheZ localization and assessed the role of regions in the N-terminal helix and four-helix bundle of CheZ on both localization changes and chemotaxis. Furthermore, a novel and widespread motif affecting monopolar localization of CheZ was identified, which might be also important for the modulation of CheZ polar localization in other Proterobacteria.

### Materials and Methods

#### Bacterial Strains and Growth Conditions

*Azorhizobium caulinodans* ORS571, its derivatives, and *E. coli* strains are listed in Table 1. *A. caulinodans* strains were grown in TY media at 37°C. *E. coli* strains were cultured in Luria broth (Luria et al., 1960) at 37°C.

#### Generation of CheZ Variants

To construct CheZ variants, a fragment including *cheZ* gene and its native promoter was amplified by polymerase chain reaction (PCR). Then an *egfp* gene encoding enhanced GFP was amplified from pEGFP-N1. The two fragments were linked by overlap extension, as previously described (Ho et al., 1989). Next, the resulting construct CheZ-GFP fusion was cloned into a broad-host-range plasmid pBBR1CMS-2 (Kovach et al., 1995), and the pBBR1-CheZ-GFP was used as a temple to construct other CheZ variants. Both the truncated mutants such as CheZ1-158-164-F and site-directed mutants such as CheZC161A-GFP were constructed by overlap extension PCR as described by Ho et al. (1989). All the CheZ variants were introduced into the *cheZ* mutant strain by triparental conjugation using a helper plasmid pRK2013 (Ditta et al., 1980). Primer pairs used in the construction are listed in Table 2.

#### Microscopy and Data Analysis

After growing in TY solid medium for overnight with shaking, cells with GFP fusion were used for observation. After growing in TY solid medium for overnight with shaking, cells with GFP fusion were used for observation.
Meier and Scharf (2009). Images were taken by an Olympus DP73 camera on an Olympus BX53 system fluorescence microscope with a 100 × objective and controlled by a cellSens Dimension 1.7 imaging software (Olympus Inc.). A space between 505 to 550 nm filter was used to detect fluorescence signals. The images analyzing spatial distribution of CheZ were processed by ImageJ1 as described by Thiem et al. (2007). Distribution of CheZAC was manually enumerated and classified into three types (diffuse, bipolar, and monopolar localization). CheZAC cells with monopolar or bipolar localization showed obvious bright spots at one end or both ends of cell. When the brightness in the whole cell distribute evenly, the localization of CheZAC in these cells is counted as diffuse. ImageJ was used to quantify the brightness at different regions of cells. Experiments were repeated at least three times, and for each sample at least 100 cells were counted.

**Soft Agar Plate Assay**

The chemotactic behavior of cheZ mutant derivative strains was assessed using soft agar plate assay, as previously described (Miller et al., 2009). Overnight bacterial cultures were washed with chemotaxis buffer at least two times and then adjusted to OD600 of 0.6. Five microliter of cells was dropped in the center of 0.3% soft agar plate. After culturing for 3 days at 37°C, the chemotactic rings on soft agar plate were counted. Ten mM sodium lactate was used as sole carbon source. Experiments were repeated at least three times.

**Sequence Alignment and Analysis**

CheZ sequences from different Proteobacteria classes were selected from Mist 2.21 (Ulrich and Zhulin, 2010) and NCBI database3. The amino acid sequences alignment was performed by an EMBL Needle program4. Secondary and tertiary structure of CheZ proteins were predicted using online server SWISS-MODEL5 and Jpred46. The phylogenetic tree was established using MEGA7 software (Kumar et al., 2016). The multiple sequences of CheZ were aligned by T-coffee7 (Notredame et al., 2000) and BioEdit (Alzohairy, 2011). Hundreds of CheZ sequences selected from NCBI database from each class of Proteobacteria were aligned by MEGA7, then the constructing file was put into Jalview (Waterhouse et al., 2009) to produce a graphical representation, and the region including phosphatase active sties was put into WebLogo (Crooks et al., 2004).

**DATA AVAILABILITY STATEMENT**

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

**AUTHOR CONTRIBUTIONS**

XL and ZX conceived and designed the experiments, analyzed the data, prepared the figures and tables, and wrote the manuscript. XL, YL, and XD carried out the experiments. KJ helped with the improvement and revision of the manuscript. All authors approved the submission for publication.

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**SUPPLEMENTARY MATERIAL**

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2020.585140/full#supplementary-material

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1 https://imagej.nih.gov/ij/
2 https://mistdb.com/
3 https://www.ncbi.nlm.nih.gov/
4 https://www.ebi.ac.uk/Tools/psa/emboss_needle/
5 https://swissmodel.expasy.org/
6 http://www.compbio.dundee.ac.uk/jpred/
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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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