Two *Agrobacterium tumefaciens* CheW Proteins Are Incorporated into One Chemosensory Pathway with Different Efficiencies

Zhiwei Huang, Qingxuan Zhou, Pan Sun, Jing Yang, and Minliang Guo[†]

College of Bioscience and Biotechnology, Yangzhou University, Yangzhou City, Jiangsu 225009, P R China

Accepted 24 November 2017.

Agrobacterium tumefaciens is the agent that causes crown gall tumor disease on more than 140 species of dicotyledonous plants. Chemotaxis of *A. tumefaciens* toward the wound sites of the host plant is the first step to recognize the host. CheW is a coupling protein that bridges the histidine kinase CheA and the chemoreceptors to form the chemotaxis core signaling complex and plays a crucial role in the assembly and function of the large chemosensory array. Unlike all previously reported chemotaxis systems, *A. tumefaciens* has only one major *che* operon but two *cheW* homologs (*atu2075* as *cheW₁* and *atu2617* as *cheW₂*) on unlinked loci. The in-frame deletion of either *cheW* gene significantly affects *A. tumefaciens* chemotaxis but does not abolish the chemotaxis, unless both *cheW* genes were deleted. The effect of *cheW₂* deletion on the chemotaxis is more severe than that of *cheW₁* deletion. Either CheW can interact with CheA and couple it to the cell poles. The promoter activity of *cheW₂* is always higher than that of *cheW₁* under all of the tested conditions. When two *cheW* genes were adjusted to the same expression level by using the identical promoter, the difference between the effects of two CheW proteins on the chemotaxis still existed. Therefore, we envision that both the different molecular ratio of two CheW proteins in cell and the different affinities of two CheW proteins with CheA and chemoreceptors result in the efficiency difference of two CheW proteins in functioning in the large chemosensory array.

Chemotaxis allows bacterial cells to modulate their swimming patterns in response to chemoeffectors and keeps them moving forward to an optimal environment, especially under adverse conditions (Dogra et al. 2012; Martin et al. 2001; Sourjik and Wingreen 2012). It not only provides a competitive advantage for motile bacteria to survive in harsh environment but also plays a key role in many biological processes such as the invasion of many bacterial pathogens into their hosts (Erhardt 2016; Falke and Piasta 2014; Sourjik and Wingreen 2012), development of bacterial biofilm (Alexandre 2015; He and Bauer 2014; Mangwani et al. 2016; Merritt et al. 2007), chemoattraction of bacteria toward

biodegradable pollutants (Adadevoh et al. 2016; Krell et al. 2013; Kudryasheva and Tarasova 2015; Mangwani et al. 2016; Montañó et al. 2013), and establishment of symbiotic association of bacteria with plants (Scharf et al. 2016).

Most environmental sensing in prokaryotes is performed by a two-component system, which typically consists of a central histidine kinase with a sensor domain and a phosphorylatable response regulator. The chemotaxis signaling transduction system is a special case of this two-component system. In the chemotaxis model bacterium *Escherichia coli*, the chemotaxis system comprises various chemoreceptors and six core components: CheA, CheW, CheY, CheB, CheZ, and CheR. In this system, the central histidine kinase CheA utilizes transmembrane chemoreceptors (usually called methyl-accepting chemotaxis proteins [MCP]) as sensors to sense the extracellular signal molecules (Bi and Lai 2015; Salah Ud-Din and Roujeinikova 2017) and the signal transduction from MCP sensor to the histidine kinase CheA requires a coupling protein, CheW, which couples CheA to the cytoplasmic part of the chemoreceptor to form a ternary signaling complex (also called a core complex) (Nishiyama et al. 2014). Hundreds of core complexes join together to form the large chemosensory array in a hexagon pattern (Falke and Piasta 2014). As a monodomain protein, CheW plays a crucial role in the assembly and function of this signaling complex (Griswold and Dahlquist 2002; Zhao and Parkinson 2006a,b). In response to the increase of repellent concentration or the reduction of attractant concentration, MCP activates CheA autophosphorylation. Phosphorylated CheA subsequently transfers its phosphoryl groups to two response regulator proteins, CheB and CheY. The phosphorylated CheY diffuses from the ternary signaling complex to the flagellar motor and changes the rotational direction of flagella from counterclockwise (CCW) to clockwise (CW) by binding to the switch component of the motor. CCW rotation is the default motor direction that produces forward swimming (“runs”), while CW rotation causes cell tumbling and changes the moving pattern (Parkinson et al. 2015). Hence, the chemotactic behavior of *E. coli* is regulated by the phosphorylation extent of CheY (Dogra et al. 2012). Phosphorylated CheY can dephosphorylate spontaneously and the chemotaxis signal is terminated. This process can be accelerated by the phosphatase CheZ (Hess et al. 1988). Phosphorylated CheB acts as a methyl-esterase to remove the methyl groups from the methylated glutamates in the signaling domain of MCP (Springer et al. 1977). Demethylation decreases MCP’s ability to activate CheA, resulting in adaptation (Bi and Lai 2015; Salah Ud-Din and Roujeinikova 2017). Chemotactic behaviors and their molecular mechanisms have been intensively studied in the γ -proteobacterium *E. coli*. For more details, please see recent reviews (Jones and Armitage 2015; Parkinson et al. 2015; Porter et al. 2011).

[†]Corresponding author: M. Guo; E-mail: guoml@yzu.edu.cn

Funding: This work was funded jointly by the National Natural Science Foundation of China (Grant Number 30870054, 31170073) and the Funding Plan for the High-level Talents with Oversea Education to Work in China from Ministry of Human Resources and Social Security of China.

*The e-Xtra logo stands for “electronic extra” and indicates that four supplementary figures and two supplementary tables are published online.

The gram-negative soilborne bacterium *Agrobacterium tumefaciens* is the causative agent of crown gall tumor disease on a broad range of dicotyledonous plants. *A. tumefaciens* causes the crown gall tumor disease by transferring a DNA fragment (called T-DNA) from its Ti plasmid to host plant cell and genetically transforming the host (Guo et al. 2009a, 2011; Subramoni et al. 2014). Its ability to mediate the interkingdom DNA transfer and the possibility to replace the oncogenes in the T-DNA with the interest genes have made *A. tumefaciens* evolve from a phytopathogen to a powerful genetic transformation tool for generating transgenic plants. Therefore, in past decades, *A. tumefaciens* has attracted the attention of a broad range of scientists, including microbiologists, phytopathologists, plant breeders, and molecular biologists. It has been extensively studied and soon became a model bacterium for the studies of many bioprocesses, which include microbe–plant interaction (McCullen and Binns 2006; Pitzschke 2013), horizontal gene transfer (Bourras et al. 2015; Matveeva and Lutova 2014; Nester 2015), type IV secretion system transport (Chandran 2013; Guo et al. 2007b; Low et al. 2014), and quorum sensing (Lang and Faure 2014; Subramoni et al. 2014). However, only a few studies on *A. tumefaciens* are related to the chemotaxis signaling mechanism, although chemotaxis is very important for *A. tumefaciens* to recognize the host and seek the invading site in bulk soil. *A. tumefaciens* is a highly motile bacterium and has a highly sensitive chemotaxis system. It can sense and be attracted toward many attractants such as sugars, amino acids, opines, and phenolic compounds that are released by host plants (Ashby et al. 1987, 1988; Kim and Farrand 1998; Loake et al. 1988). *A. tumefaciens* commonly lives in the rhizosphere (Escobar and Dandekar 2003) and the invasion of *A. tumefaciens* into host plant begins with its chemotaxis toward the wound site of the host (Ashby et al. 1987; Hawes and Smith 1989; Hawes et al. 1988; Parke et al. 1987) but the chemotaxis signaling transduction pathway in *A. tumefaciens* remains unclear.

Most plant-associated bacteria, which include species or strains from the genera *Azorhizobium*, *Azospirillum*, *Bradyrhizobium*, *Rhizobium*, and *Sinorhizobium*, have multiple chemotaxis systems (Scharf et al. 2016), whereas, with the exception of most *mcp* and two *cheW* genes, the *A. tumefaciens* C58 genome encodes only one complete set of core chemotaxis proteins and all of the core component-encoding genes are organized into one *che* operon (Goodner et al. 2001; Guo et al. 2017; Wood et al. 2001; Wright et al. 1998). Deletions of *orf1*,

cheA, *cheY1*, *cheY2*, *cheB*, and *cheR* in this *che* operon impaired the chemotaxis of *A. tumefaciens* toward acetosyringone (AS) (Harighi 2008, 2009; Wright et al. 1998). Unlike the *che* operon in many bacteria, the *A. tumefaciens che* operon does not have the *CheW*-encoding gene, and two *cheW* homologs (*atu2075* as *cheW₁* and *atu2617* as *cheW₂*) are found at different loci of the circular chromosome (Goodner et al. 2001; Wood et al. 2001). Until now, the roles of these two *cheW* homologs in chemotaxis have not been identified. In this study, two *cheW* genes of *A. tumefaciens* were precisely deleted by gene replacement, and their roles in chemotaxis were investigated by a series of experiments. Our results revealed that two *CheW* proteins coexist in one chemosensory pathway and both *CheW* proteins are required for the full chemotaxis signaling transduction in *A. tumefaciens*; however, the effect of *cheW₂* on chemotaxis is more severe than that of *cheW₁*.

RESULTS

Two *CheW* proteins are very similar in structure.

In the *A. tumefaciens* genome, two *cheW* genes are located at different loci on the circular chromosome rather than in the *che* operon (Goodner et al. 2001; Wood et al. 2001). There is no putative chemotaxis or motility gene adjacent to *cheW₁* (*atu2075*). Only one putative MCP-encoding gene (*atu2168*) is located downstream of *cheW₂* (*atu2167*). Therefore, the bioinformatics analysis of two *CheW* proteins is useful for us to further verify the annotation of two *cheW* genes and get more information about the functions of these two putative *CheW* proteins. According to the prediction in the genome database, the peptide lengths encoded by *cheW₁* and *cheW₂* genes are 155 and 159 amino acid residues, respectively. Sequence alignment with the *CheW* from *E. coli* showed that 30.66% of the *CheW₁* sequence and 32.68% of the *CheW₂* sequence are identical with the sequence of *E. coli CheW* (Fig. 1). The sequence alignment also showed that both *CheW₁* and *CheW₂* contain most of the highly conserved residues, which are identified to be involved in the *CheW*–MCP and *CheW*–*CheA* interactions in *E. coli CheW* (Alexandre and Zhulin 2003; Boukhvalova et al. 2002a,b; Liu and Parkinson 1989, 1991; Piñas et al. 2016). Due to the high similarity of two *A. tumefaciens CheW* proteins with *E. coli CheW*, the solution structure of *E. coli CheW* (Li et al. 2007) was used as the structure template to model the structures of these two *A. tumefaciens CheW* proteins. The homology models of two *A. tumefaciens CheW* proteins manifested

Fig. 1. Sequence alignment of *Escherichia coli CheW* and two *Agrobacterium tumefaciens CheW* proteins. Numbers on the right indicate the position of amino acid. Sequences of two *CheW* proteins are from the University of Washington version of the genome data (Wood et al. 2001). Asterisks represent the key residues that are identified to be involved in the *CheW*–methyl-accepting chemotaxis protein and *CheW*–*CheA* interactions in *E. coli CheW* (Alexandre and Zhulin 2003; Boukhvalova et al. 2002a,b; Liu and Parkinson 1989, 1991). Inverted triangles indicate the key residues of *E. coli CheW* for functioning interface 2 interaction between *CheW* and the P5 domain of *CheA* (Piñas et al. 2016).

that the structures of both *A. tumefaciens* CheW proteins are very similar to the structure of *E. coli* CheW, with root-mean-square deviations of backbone atoms less than 0.148 Å.

Two *cheW* genes are expressed simultaneously in the same cell.

Effective discrimination of two CheW homologous proteins is the prerequisite condition for the study of the expressions of two *cheW* genes. CheW₁ shares 47.17% sequence identity with CheW₂ (Supplementary Fig. S1). If the full-length CheW proteins were used to generate polyclonal antibodies, the antibodies would be likely to cause cross reaction between two CheW proteins and lead to nonspecific results. Therefore, the peptide fragments from the variable sequence regions of these two CheW proteins (142 to 155 amino acid residues of CheW₁ and 146 to 159 amino acid residues of CheW₂) were synthesized and used as antigens to generate the polyclonal rabbit antibodies that could differentiate two CheW proteins without cross reaction. Two CheW proteins that are heterogeneously produced by *E. coli* BL21 (DE3) can be specifically identified by their corresponding antibodies (Fig. 2), demonstrating that the antibodies are able to discriminate between two CheW proteins and, thus, can be used to test the expressions of two *cheW* genes. To investigate the expressions and functions of *cheW*₁ and *cheW*₂ in *A. tumefaciens*, two *cheW* genes were precisely deleted by homologous recombination (as described in Materials and Methods). Two single mutants of these two *cheW* genes were named C58Δ*W*₁ and C58Δ*W*₂. The double-mutant was named as C58Δ*W*. To restore the synthesis of CheW proteins in the mutants, *cheW* genes with their native promoters (approximately 500 bp upstream of the *cheW* gene) were reintroduced to the corresponding mutants by using plasmids. The complemented mutant strains were named C58Δ*W*₁+, C58Δ*W*₂+, and C58Δ*W*+. The expressions of two *cheW* genes in different strains were verified by Western blot. Immunoblotting results showed that both CheW₁ and CheW₂ can be detected in the wild type and all of the complemented mutants, demonstrating that both *cheW* genes can be expressed simultaneously in the same cell and the complemented *cheW* genes can also be expressed in the mutant strains (Fig. 2). According to the band strengths of two CheW proteins in different strains, the deletion of either *cheW* gene does not affect the expression of another *cheW* gene, and both CheW proteins expressed by plasmid show increased levels compared with the wild type. Results also showed that the corresponding CheW proteins in the mutant strains cannot be detected by the Western blot (Fig. 2), confirming that the corresponding *cheW* genes are deleted.

Two *cheW* genes have different effects on *A. tumefaciens* chemotaxis.

A. tumefaciens has only one chemotaxis signaling pathway. CheW bridges CheA to MCP and transfers the signal from

different MCP to CheA. What we wanted to know was why *A. tumefaciens* expresses two CheW proteins for one chemotaxis signaling pathway and whether the two CheW proteins have different functions. To answer these questions, we characterized the chemotaxis behaviors of *cheW*-deletion mutants. Swimming in swim agar plate and attraction toward plant leaf disc were used to evaluate the effects of *cheW* deletions on *A. tumefaciens* chemotaxis. When *A. tumefaciens* cells were inoculated on the swim agar plate, the cells utilized the nutrients, thus resulting in a nutrient concentration gradient. Bacterial cells with chemotaxis will steadily move outward along the nutrient gradient, thus growing a big colony. The colony size on the swim agar plate is often used to indicate the chemotaxis behaviors of bacteria to nutrient gradient (Sampedro et al. 2015). Because the *A. tumefaciens* genome possesses only one CheA-encoding gene and CheA is the key player of chemotaxis signaling transduction, the deletion of the *cheA* gene will be able to abolish the chemotaxis signaling transduction in *A. tumefaciens*. Therefore, the *cheA*-deletion mutant of *A. tumefaciens* can be used as a negative control of chemotaxis. The colony size of the double mutant C58Δ*W* is the same as that of the *cheA*-deletion mutant C58Δ*A* (Fig. 3A and B), demonstrating that the deletion of both *cheW* genes can fully eliminate the chemotaxis signaling transduction and *A. tumefaciens* does not have any other gene that can partially compensate the functions of two *cheW* genes. Colonies of both the C58Δ*W*₁ and C58Δ*W*₂ mutants were smaller than that of wild-type C58 and the colony of C58Δ*W*₂ mutant was smaller than that of the C58Δ*W*₁ mutant (Fig. 3A and B), implying that the deletion of either *cheW* gene is able to weaken the chemotaxis, and that the effect of *cheW*₂ deletion on the chemotaxis is more severe than that of *cheW*₁ deletion. To verify whether the deletions of *cheW* genes or *cheA* would affect the growth rate of *A. tumefaciens*, resulting in the difference of these *cheW* mutants in colony size, we tested the growth curves of these mutants in AB-sucrose liquid medium. Growth curves showed that the growth rates of these *A. tumefaciens* mutants in AB-sucrose liquid medium were the same as that of the wild-type strain C58 (Supplementary Fig. S2).

Chemotaxis of *A. tumefaciens* toward attractants can be characterized by the attraction of agrobacterial cells toward a *Kalanchoe* leaf disc (Su et al. 2006). The leaf disc, which is placed at the center of the swim agar plate, will produce exudates and create an exudate concentration gradient from the plate center to the plate edge. Bacterial cells that can be attracted by the exudates will move toward the plate center along the exudate gradient. The chemotaxis was quantitated by relative migration distance, which was defined as the difference of migration distance between toward and away from the leaf disc (Fig. 3C and D). The results showed that the chemotaxis behaviors of all the *A. tumefaciens* strains tested with plant wound exudates were similar to those tested with nutrient substances (Fig. 3C and D); in particular, the

Fig. 2. Western blot analysis of *cheW* mutants and their complemented strains. Equal amounts of cell crude extracts from different bacterial strains were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis and then analyzed by immunoblotting. CheW₁ = crude extract from the *Escherichia coli* BL21 (DE3) cells overexpressing the *cheW*₁ gene and CheW₂ = crude extract from the *E. coli* BL21 (DE3) cells overexpressing the *cheW*₂ gene. Antibodies used for immunoblotting are listed on the right. Other labels on the top of the lanes represent the *Agrobacterium tumefaciens* strains where the crude extracts originated.

relative migration distance of the C58 Δw_2 mutant was smaller than that of C58 Δw_1 mutant. Bacteria use different MCP to sense different chemoeffectors. The chemotactic response of the C58 Δw_2 mutant was always weaker than that of the C58 Δw_1 mutant no matter what chemoeffectors they responded to, implying that the effects of the two CheW proteins on the chemotaxis signaling transduction do not depend on the MCP.

Two CheW proteins can transfer the signal from the same MCP.

To know whether the two CheW proteins can recognize the same MCP and transfer the signal from the same MCP to CheA, we tested the chemotactic responses of *A. tumefaciens* C58 *cheW*-mutants to a single chemoattractant, AS. AS is a strong chemoattractant to *A. tumefaciens* (Ashby et al. 1987; Stachel et al. 1985). To test the chemotaxis of bacteria toward a single chemoattractant requires a more sensitive method. Therefore, we used the oldest method for measuring chemotaxis, the capillary assay (Adler 1973; Shaw 1995), to determine the chemotactic responses of *A. tumefaciens* C58 *cheW*-deletion mutants to AS. Deficiency of either CheW significantly attenuated the chemotactic response of *A. tumefaciens* to AS and *A. tumefaciens* required both CheW proteins to completely transfer the chemotaxis signal (Fig. 4). These results suggested that two CheW proteins are able to recognize the same MCP and transfer the signal from

the same MCP to CheA, although two CheW proteins may transfer the signal with different efficiencies, implying that two CheW proteins are not responsible for recognizing different MCP; instead, both CheW proteins can transfer the signal from the same MCP and are incorporated into one chemotaxis signaling pathway. Results also showed that the effect of *cheW*₂ deletion on the attraction of agrobacterial cells toward AS is more severe than that of *cheW*₁ deletion (Fig. 4).

Both CheW₁ and CheW₂ interact with CheA.

Most MCP are transmembrane chemoreceptors and located at the cell poles. CheW acts as an adapter protein to tether CheA to different MCP, forming ternary signaling complexes at the cell poles (Nishiyama et al. 2014; Parkinson et al. 2015; Sourjik and Berg 2000). CheW is essential for the assembly of these ternary complexes. Therefore, we wanted to know whether the two CheW proteins had any significant difference in assembling the ternary complex. To visualize the cellular localization of CheA, an *egfp-cheA* fusion gene expression cassette was introduced into the C58 Δa , C58 Δw_1 , C58 Δw_2 , and C58 Δw mutant strains by using plasmid. The *egfp-cheA* fusion gene in the plasmid can be expressed by *A. tumefaciens* into an eGFP-CheA fusion protein, in which enhanced green fluorescent protein (eGFP) is fused onto the N terminus of CheA. It is important to know whether the eGFP-CheA fusion protein still

Fig. 3. Chemotactic responses of *Agrobacterium tumefaciens* C58 *cheW*-deletion mutants to nutrient substances and plant attractants. Equal amounts of the cells from different *A. tumefaciens* strains that were grown to middle-log phase were inoculated on the swim agar plate. **A** and **B**, The radius of the bacterial colony after growing for 48 h on the swim agar plate was used to quantitate the chemotactic response of *A. tumefaciens* to nutrient substances. For the test of the chemotactic responses of *cheW*-deletion mutants to plant attractants, a disc of *Kalanchoe* leaf was placed at the center of the swim agar plate to produce exudate attractants. Each inoculating spot had equal distance from the central leaf disc, so that each strain could be inoculated on the same attractant gradient. **C** and **D**, Chemotaxis toward plant attractants was quantitated by the relative migration distance. Relative migration distance = the distance between the edge toward the leaf disc and inoculating spot – the distance between the edge away from the leaf disc and the inoculating spot. **A** and **C**, Typical colonies of different *A. tumefaciens* strains after growing for 48 h on the swim agar plate without or with plant leaf disc. **B**, Colony radii of different *A. tumefaciens* strains after growing for 48 h on the swim agar plate without leaf disc. **D**, Relative migration distance of different *A. tumefaciens* strains after growing for 48 h on the swim agar plate with leaf disc. Data are the means from three plates with standard deviations (**B** and **D**). Asterisks (*) represent *P* values of these strains <0.01 compared with the wild type strain C58. Labels on the plate images (**A** and **C**) and the horizontal axes (**B** and **D**) are the names of the corresponding *A. tumefaciens* strains.

has the function of native CheA. Fortunately, the eGFP-CheA fusion protein was able to restore the chemotactic response of the C58 Δ mutant (Supplementary Fig. S3). The observation of the fluorescence of these mutant strains showed that the eGFP-CheA fusion protein is located at the cell poles in all of the *cheA*-deletion mutant strains with either or both of the *cheW* genes but uniformly distributed throughout whole cell in the *cheA*-deletion mutant with neither of the *cheW* genes (Fig. 5). These results confirmed that both CheW proteins can couple CheA to MCP and form a stable ternary complex but did not show any discernable difference between two CheW proteins in coupling CheA to MCP.

An in vitro pull-down experiment was used to further verify the interaction between CheA and CheW. A his-tag was fused onto the C terminus of CheA to form a CheA-His fusion protein. Another his-tagged fusion protein, Salt-His, which has nothing to do with chemotaxis, was used as a negative control. CheA-His and Salt-His fusion proteins, which were over-produced by *E. coli*, were bound to Ni-IDA resin. The Ni-IDA resins bound with CheA-His or Salt-His were incubated with the crude extract from C58 Δ strain to test whether the CheA-His fusion protein can pull down both CheW₁ and CheW₂ from the crude extract. Both CheW₁ and CheW₂ were detected in the eluate from the resin bound with CheA-His (Fig. 6, lane 1) but not in the negative control (Fig. 6, lane 2), confirming that both CheW₁ and CheW₂ can interact with CheA in vitro.

The difference of the effects of *cheW*₁ and *cheW*₂ on chemotaxis arises from both the promoter activity and the protein itself.

According to the above results, the deletion of either *cheW* gene can weaken the chemotaxis behavior and both *cheW*₁ and *cheW*₂ are required for the full chemotaxis but their effects on chemotaxis are different. Therefore, we wanted to know the cause of the different effects. *cheW*₁ (*atu2075*) and *cheW*₂ (*atu2617*) are located in different operons and controlled by

Fig. 4. Chemotactic responses of *Agrobacterium tumefaciens* C58 *cheW*-deletion mutants to acetosyringone (AS). Cells of different *A. tumefaciens* strains, which were grown to middle-log phase, were collected, washed, and resuspended in chemotaxis medium to an optical density at 600 nm of 0.1. Capillary tubes containing AS at 10⁻⁷ mol/liter were inserted into the agrobacterial cell suspensions for 1 h at room temperature (25°C). Then, the liquid in the capillary tube was transferred, diluted proportionately, and plated in MG/L solid medium for colony count. The results are expressed as the means $\pm$ standard error of the mean from five capillary tubes. Asterisks (*) in the figure represent *P* values of these mutants <0.01, compared with the chemotaxis-negative strain (C58 Δ a).

different promoters. Therefore, the expression levels of *cheW*₁ and *cheW*₂ may be one of the reasons that lead to their different effects on chemotaxis. To determine the promoter activities of two *cheW* genes, a red fluorescent protein (RFP)-encoding gene (*rfp*) was used as a reporter gene for the in situ substitute for *cheW*₁ and *cheW*₂. The expression level of the reporter gene *rfp*, which is in situ controlled by the promoter of *cheW*₁ or *cheW*₂, can be quantitated by the RFP fluorescence intensity. Expression of the *rfp* gene under the in situ control of *cheW*₁ or *cheW*₂ promoter is shown in Figure 7. The fluorescence intensity of the crude extract from the strain, in which the *rfp* gene is in situ controlled by *cheW*₂ promoter, is always higher than that of the crude extract from the strain, in which the *rfp* gene is in situ controlled by *cheW*₁ promoter, implying that the promoter activity of *cheW*₂ is stronger than that of *cheW*₁. This means that the concentration of CheW₂ is always higher than that of CheW₁ in *A. tumefaciens* cells, although both the absolute concentrations and the relative ratio of the two CheW proteins at different growth times may be regulated by the promoters of the two *cheW* genes.

To determine whether the two CheW proteins themselves have different effects on chemotaxis, we compared the effects of the two *cheW* genes on chemotaxis under the control of an identical promoter. When two *cheW* genes were controlled by the identical promoter, the concentrations of the two CheW proteins in the cells would be the same. If two *cheW* genes had different effects on chemotaxis under the control of an identical promoter, the difference of the effects should be ascribed to the CheW proteins themselves. To be able to express two *cheW* genes in *A. tumefaciens* cells under the control of an identical promoter, the promoters of *cheW*₁ and *cheW*₂ were exchanged with each other. Generally, a prokaryotic promoter and its regulatory elements are located in the region approximately 500 bp upstream of the gene (Browning and Busby 2016; Minchin and Busby 2009). We constructed four plasmids, which carry four different *cheW* expression cassettes: 479 bp upstream of the *cheW*₁ + *cheW*₁ open reading frame (ORF), 520 bp upstream of the *cheW*₂ + *cheW*₁ ORF, 479 bp upstream of the *cheW*₁ + *cheW*₂ ORF, and 520 bp upstream of the *cheW*₂ + *cheW*₂ ORF. The four plasmids were introduced into the C58 Δ W mutant and the chemotaxis behaviors of these four C58 Δ W strains harboring different plasmids were compared. *cheW*₁ and *cheW*₂ had different effects in restoring the chemotaxis behaviors of the C58 Δ W strain even if both *cheW*₁ and *cheW*₂ were under the control of the identical promoter (Fig. 8). To verify whether two CheW proteins have the same expression level under the control of the same promoter, Western blot was used to evaluate the concentrations of two CheW proteins in the cells of different agrobacterial strains. In order to adjust the possible titer difference of two anti-CheW antibodies, two His-tagged CheW fusion proteins (CheW₁-His and CheW₂-His), which were expressed under the control of the same promoter, were used as the reference proteins for Western blot. Western blot results confirmed that the actual expression levels of CheW₁ and CheW₂ are the same when they are expressed under the control of the same promoter (Supplementary Fig. S4). This implies that the expression products of the two *cheW* genes, CheW₁ and CheW₂ proteins, have different effects on *A. tumefaciens* chemotaxis in the same concentration. Results also showed that the efficiencies of the two CheW proteins to restore the chemotaxis behaviors of the C58 Δ W strain are dependent on what chemoreceptors they couple CheA to (Fig. 8).

DISCUSSION

CheW, as an adaptor protein, not only bridges CheA to MCP to form a ternary core signaling complex but also couples the

kinase control domain of MCP to modulate the kinase activity of CheA (Nishiyama et al. 2014; Parkinson et al. 2015). CheW is essential to chemotaxis (Parkinson and Houts 1982). Although the model organism of chemotaxis, *E. coli*, has a single coupling protein (CheW), multiple CheW homologs are universal in many bacteria such as *Rhodobacter sphaeroides* (Martin et al. 2001), *Leptospira interrogans* (Li et al. 2006), *Vibrio cholerae* (Butler et al. 2006), and *Azospirillum brasiliense* (Scharf et al. 2016). According to previous studies, multiple *cheW* homologs include the following four cases. (i) Multiple *cheW* homologs are located in different chemotaxis operons. Different *cheW* genes are under distinct transcriptional control and induced by different environmental factors. Therefore, different CheW proteins mediate different chemotaxis pathways under different lifestyles and only conditionally affect the chemotactic responses (Martin et al. 2001). (ii) Multiple *cheW* homologs are located on different loci. Multiple CheW homologs are incorporated into one chemotaxis signaling pathway and any of these CheW proteins is essential for chemotaxis (Zhang et al. 2012). (iii) Multiple *cheW* homologs are located on the distinct unlinked loci. Only one of the CheW homologs is essential for chemotaxis. Other CheW homologs are dispensable or functionally redundant for chemotaxis (Butler et al. 2006). (iv) One CheW with multiple homologs of a related protein (CheV) coexists in the chemotaxis signaling network. CheV consists of a CheW domain fused to a phosphorylatable receiver domain and has CheW function in the

N terminus and response-regulator CheY function in the C terminus (Lowenthal et al. 2009; Ortega and Zhulin 2016; Pittman et al. 2001). Multiple *cheV* homologs are located on the distinct unlinked loci (Abedrabbo et al. 2017; Alexander et al. 2010; Pittman et al. 2001; Rosario et al. 1994). CheW and CheV have redundant abilities to interact with MCP and CheA but both CheW and CheV are required to modify the formation of a large chemosensory array and, thus, provide flexibility in the large chemosensory array formation (Abedrabbo et al. 2017; Pittman et al. 2001). In our results, both *A. tumefaciens cheW* genes that are located on two distinct, unlinked loci are required for optimal chemotaxis but neither of them is absolutely necessary for chemotaxis. CheW₁ and CheW₂ possess largely overlapping or redundant but not identical functions in the chemotactic behavior of *A. tumefaciens*. This situation of two *cheW* homologs in *A. tumefaciens* is different from any of the four previously reported cases, suggesting that the two CheW proteins use a unique mechanism to incorporate into one chemotaxis signaling pathway.

CheW promotes the formation of the MCP-CheW-CheA ternary complex by interacting with both MCP and CheA. The smallest assembly of the ternary core signaling complex contains two MCP trimers-of-dimers, two molecules of CheW, and one CheA homodimer. The minimal core complexes are linked into hexagonal arrays through a unique interface 2 interaction between CheW and the P5 domain of CheA to form large chemosensory arrays at cell poles, producing highly cooperative

Fig. 5. Cellular localization of CheA in *Agrobacterium tumefaciens* C58 *cheW*-deletion mutants. Plasmid pUCA-19GA, which carries an *egfp-cheA* fusion gene expression cassette, was introduced into different *A. tumefaciens* strains. *A. tumefaciens* strains harboring this plasmid were grown to middle-log phase. Cells of these strains were observed by using confocal laser-scanning microscopy. **A**, eGFP-CheA in C58Δa cells. **B**, eGFP-CheA in C58Δaw₁ cells. **C**, eGFP-CheA in C58Δaw₂ cells. **D**, eGFP-CheA in C58Δaw cells. White bars correspond to 3 μm.

signaling properties (Bi and Lai 2015; Briegel and Jensen 2017; Piñas et al. 2016; Salah Ud-Din and Roujeinikova 2017). The array cooperativity relies on coupling between core complexes through the unique interface 2. Residues R117, E121, and F122 in the *E. coli* CheW are the key interface 2 residues (Piñas et al. 2016). However, the residues of two *A. tumefaciens* CheW proteins in this interface 2 region show very low similarity (Fig. 1), implying that two CheW proteins may have different affinities to interact with CheA in the interface 2. The difference of the affinities of two CheW proteins to CheA would affect the coupling of CheA and core complex and, thus, the cooperative efficiency of the large chemosensory array. This may explain why two *cheW* genes still show different abilities to restore the chemotaxis of the *cheW* double-mutant when their actual expressions are adjusted to the same level by the same promoter. Both the concentration of free CheW and the affinity of CheW to CheA affect the incorporation of CheW into the large chemosensory array, resulting in the variation of the ratio of CheW₁ to CheW₂ in the large chemosensory array. Finally, the ratio of CheW₁ to CheW₂ in the large chemosensory array affects the cooperative efficiency of the large chemosensory array in signaling transduction and, thus, affects the chemotactic response. It should be pointed out that excessive CheW can disrupt the chemoreceptor signaling array in *E. coli* (Cardozo et al. 2010). In our results, the superficial contradiction between the results in Figure 8 and those from Figures 3, 4, and 7 also revealed that the effects of two *A. tumefaciens* CheW proteins on chemotactic response are dependent on their expression levels. Therefore, the determination of the actual affinities of two CheW proteins to CheA and different MCP will be helpful for us to calculate the normal stoichiometries of CheA, two CheW proteins, and MCP in the large chemosensory array and to quantitate the efficiencies of two CheW proteins in transducing the chemotaxis signal. In addition, the *A. tumefaciens* mutant with a single MCP will be useful for the understanding of the mechanism of the interaction between MCP and two CheW proteins because *A. tumefaciens* C58 possesses 20 MCP.

MATERIALS AND METHODS

Plasmids, bacterial strains, and their growth conditions.

Strains of *E. coli* and *A. tumefaciens* and the plasmids used in this study are listed in Supplementary Table S1. *E. coli* strains

Fig. 6. In vitro analysis of the interactions between CheA and two CheW proteins of *Agrobacterium tumefaciens*. Interactions between CheA and two CheW proteins were detected by using a His-tag pull-down assay. CheA-His fusion protein overexpressed in *Escherichia coli* was bound to Ni-IDA resin. Then, the fusion protein-bound resins were incubated with the crude extract of C58Δa to bind CheW₁ and CheW₂. Unbound proteins were washed away. Bound proteins were collected and separated on the 12% (wt/vol) sodium dodecyl sulfate polyacrylamide gel electrophoresis. CheW₁ and CheW₂ were detected by Western blot. A Salt-His fusion protein, which is unrelated to the chemotaxis, was used as a negative control and was conducted in the pull-down assay in parallel. Lane 1, the eluate from the resins containing CheA-His; lane 2, the eluate from the resins containing Salt-His as the negative control; lane 3, the crude extract of C58Δa as the positive control.

were grown in lysogeny broth liquid or agar medium at 37°C (Sambrook et al. 1989). *A. tumefaciens* strains were grown in MG/L or AB-sucrose liquid or agar medium at 28°C (Cangelosi et al. 1991; Gelvin 2006). Antibiotic concentrations were used for *E. coli* at final concentrations of ampicillin at 100 μg/ml and kanamycin at 50 μg/ml and, for *A. tumefaciens*, kanamycin at 100 μg/ml and carbenicillin at 100 μg/ml.

DNA manipulations.

DNA manipulations followed the standard molecular protocols (Sambrook et al. 1989). *A. tumefaciens* DNA was prepared according to Charles and Nester (1993). Plasmid DNA was purified with TIANprep Mini Plasmids Kit (Tiangen Biotech Corporation). Primer information is listed in Supplementary Table S2. PCR products and DNA fragments were purified from agarose gels using TaKaRa MiniBEST Agrose Gel DNA Extraction Kit (ver. 4.0; TaKaRa Corporation). *E. coli* competent cells were prepared according to the Inoue protocol and DNA was transferred into *E. coli* competent cells by heat-shock (Sambrook et al. 1989). Plasmids were introduced into *A. tumefaciens* by electroporation (Cangelosi et al. 1991).

Construction of deletion or substitution mutants of *A. tumefaciens*.

The precise deletion or substitution of *A. tumefaciens* gene *cheW*₁, *cheW*₂, or *cheA* was constructed by the gene replacement procedure, as described previously (Guo et al. 2007a, 2009b). All of the plasmids that were used for these gene replacements were derived from the plasmid pEX18Km, which carried a kanamycin-resistant gene as the positive selection marker and a suicide gene (*sacB*) as the counter-selectable marker. All *A. tumefaciens* mutants generated by this gene replacement system were verified by PCR and DNA sequencing.

Complementation construction of *cheW* or *cheA* mutants.

The complementation of the *cheW* or *cheA* mutants was implemented by the introduction of plasmids that carried the

Fig. 7. Promoter activity analysis of two *cheW* homologs. The open reading frames of two *cheW* genes in *Agrobacterium tumefaciens* strain C58 were in situ substituted by a red fluorescent protein (*rfp*) gene open reading frame, so that *rfp* gene could be used as a reporter gene to detect the activities of the two *cheW* promoters in situ. Two *A. tumefaciens* strains, C58RS1 (*cheW*₁ was substituted for *rfp*) and C58RS2 (*cheW*₂ was substituted for *rfp*), were grown for the indicated time. Cells were collected in the indicated growth time. Cell crude extracts were used for the determination of fluorescence intensity. Fluorescence intensity data were normalized with the crude extract from wild-type cells to eliminate the interference of background fluorescence signals. Each experiment was performed at least in triplicate.

cheW or *cheA* expression cassettes. To construct two *cheW* expression plasmids, DNA fragments of two wild-type *cheW* genes with their native promoters (approximately 500 bp upstream of the start code) were amplified by PCR from C58 genomic DNA and inserted into plasmid pCB301. Thus, two *cheW* expression cassettes were promoted by their native promoters. To construct *cheA* expression plasmid, only the *cheA* coding sequence was amplified and inserted downstream of the *lacZ* promoter of the plasmid pUCA-19 so that the *cheA* expression cassette could be promoted by the *lacZ* promoter. PrimeSTAR Max DNA polymerase for PCR was purchased from TaKaRa Corporation. Plasmids carrying the corresponding *cheW* or *cheA* expression cassettes were verified by restriction enzyme digestion and sequencing prior to electroporation into the corresponding mutants.

Chemotaxis assays.

Chemotactic responses to nutrient substances were assayed in AB-sucrose medium containing 0.2% Bacto agar (Sangon Biotech Co., Inc.). Briefly, cell cultures in the middle-logarithmic growth phase were normalized to an optical density at 600 nm (OD_{600nm}) of 0.5. Equal amounts (3 μ l) of each strain were inoculated on the swim agar plate and incubated at 28°C for 48 h without disturbance for the incubation period. Radii of the colonies were measured and used to quantitate the chemotaxis toward nutrient substances (Sampedro et al. 2015).

Chemotactic responses to plant attractants were assayed as described by Su et al. (2006). Briefly, the fresh leaf disc (diameter = 5 mm) of a *Kalanchoe* plant was placed at the center of the swim agar plate containing 0.2% Bacto agar. The strains were inoculated onto the plate 2.5 cm from the center of the leaf disc. Then, the plates were incubated at 28°C without disturbance and observed throughout a 48-h incubation period. Chemotactic response to AS was determined by the more sensitive capillary assay (Adler 1973; Shaw 1995). AS was purchased from Sangon Biotech Co., Inc.. The procedure of capillary assay was followed as described by Adler (1973), with minor modifications. Agrobacterial cells were harvested by centrifugation at 4,000 rpm for 3 min at room temperature (25°C) and suspended in chemotaxis medium (EDTA at 0.1 mmol/liter and KH_2PO_4 at 10 mmol/liter, pH 7.0) to an OD_{600nm} of 0.1. Agrobacterial suspension (300 μ l) was used to make a bacterial pond. The capillary tube was sealed at one end and filled with AS at 10^{-7} mol/liter dissolved in chemotaxis medium. The open end of the capillary tube was inserted into the bacterial pond and incubated for 1 h at room temperature; then, the solution in the capillary tube was expelled and completely transferred into 1 ml of AB-sucrose medium. Dilutions were plated in duplicate on MG/L plates and incubated for 2 days at 28°C. The colonies in the plate were counted and represented the number of the cells attracted to the capillary tube.

Fig. 8. Effects of two *cheW* open reading frames (ORF) under the control of identical promoters on chemotaxis. Four plasmids carrying different *cheW* expression cassettes were introduced into strain C58Δw to generate four *Agrobacterium tumefaciens* strains: C58ΔwP1W1 (*cheW*₁ ORF controlled by its own promoter), C58ΔwP2W1 (*cheW*₁ ORF controlled by *cheW*₂ promoter), C58ΔwP2W2 (*cheW*₂ ORF controlled by its own promoter), and C58ΔwP1W2 (*cheW*₂ ORF controlled by *cheW*₁ promoter). Chemotactic responses of these four *A. tumefaciens* strains to **A** and **C**, nutrient substances and **B** and **D**, plant attractants were tested. Each experiment was performed in triplicate. Asterisks (*) represent *P* value < 0.01 between the two compared strains.

Pull-down assay for detecting the interaction between CheA and CheW proteins.

E. coli BL21 (DE3) was utilized as the host to overproduce His-tagged CheA. Another His-tagged fusion protein, SalT-His, which is unrelated to the chemotaxis, was used as the negative control. The productions of His-tagged fusion proteins by *E. coli* followed the previous procedures (Gao et al. 2013; Yu et al. 2015). *E. coli* BL21 cells harboring the corresponding recombinant pET-30a plasmids were grown to approximately 5×10^8 cell/ml, and isopropylthio- β -D-galactoside was then added to the cultures at a final concentration of 0.3 mM to induce the expression of the fusion proteins. After cultivation at 25°C for 4 h, the cells were harvested by centrifugation ($6,000 \times g$, 10 min, 4°C), washed twice with phosphate-buffered saline (PBS) buffer (10 mM, pH 7.4), and resuspended in a small volume of PBS buffer. All of the following procedures were carried out at 4°C. The harvested cell suspension was sonicated to near clarity and the supernatant in the sonicated cell suspension was collected by centrifugation ($12,000 \times g$ for 30 min). The supernatants from different samples were slowly added into 1.5 ml of ProteinLoS Ni-IDA Resins (Transgen Biotech) to allow the His-tagged fusion proteins to bind the resins. The fusion protein-bound resins were washed twice with PBS buffer (10 mM, pH 7.4), then incubated with 4 ml of cell crude extract from 100 ml of cell culture of the C58 Δ a strain, which was grown to $OD_{600nm} = 1$ in MG/L liquid medium. After incubation overnight at 4°C with gentle shaking, the resins were washed with 10 resin volumes of PBS buffer containing different concentrations of imidazole (10, 30, and 50 mM) in the order of concentration increase to wash out the nonspecifically bound proteins. Finally, the specifically bound proteins on the resins were eluted with 500 μ l of PBS buffer containing 250 mM imidazole.

Generation of polyclonal rabbit antibodies against CheW₁ and CheW₂.

Polyclonal rabbit antibodies against two CheW proteins were provided by GenScript Corporation. To obtain the polyclonal antibodies that can discriminate the two CheW proteins, peptide fragments from the variable sequences of the two CheW proteins were artificially synthesized and used as the antigens to generate antibodies against the two CheW proteins in New Zealand rabbits. To test whether the polyclonal antibodies can discriminate between the two CheW proteins, *E. coli* BL21 (DE3) was used as the host to heterogeneously produce the individual CheW, so that it was impossible for two CheW proteins to coexist in one strain. The specificities of the antibodies against the two CheW proteins were verified by the heterogeneously produced individual CheW using Western blot.

Electrophoresis and immunoblotting analyses.

Electrophoresis and immunoblotting analyses were carried out as described before (Yang et al. 2015). For sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), the harvested bacterial cells from different samples were washed twice in PBS buffer (10 mM, pH 7.4) and resuspended to the same cell density. Equal amounts of cell suspensions from these different samples were mixed with 6 $\times$ protein loading buffer and boiled for 6 min. Equal volumes of the whole-cell lysates from different cell suspensions were loaded for SDS-PAGE. For immunoblotting, proteins were electrophoretically transferred to 0.22- μ m pore size hydrophilic polyvinylidene difluoride membranes (Merck Millipore) and were detected with the BCIP/NBT alkaline phosphatase color development kit (Beyotime Biotechnology Corp.), according to the procedure provided by the manufacturer. Polyclonal antibodies against the

variable sequence regions of two CheW proteins were used as the primary antibodies for detection.

Confocal laser microscopy observation of agrobacterial cells.

For microscopy observation, agrobacterial cells from midlog-phase cultures were added onto the center of the slides. A coverslip was placed on top of the culture droplet. The edges of the coverslip were sealed with acrylic polymer to prevent drying. *A. tumefaciens* cells were visualized by a Leica TCS SP8 STED 3X system using an Ar laser (excitation wavelength of 488 nm and emission wavelength of 500 to 550 nm) and a $\times 100$ oil immersion objective. The images were analyzed and edited using LAS X Core Offline, version 3.0.2.

Determination of fluorescence intensity.

A RFP, TurboRFP, was used as the reporter protein to report the activity of the *cheW*₁ or *cheW*₂ promoter. The red fluorescence intensity in the agrobacterial cell lysate supernatant was used to quantitate the expression level of the *rfp* gene under the control of the *cheW*₁ or *cheW*₂ promoter. The procedure for the collection of cell lysate supernatant and the determination of fluorescence intensity is as follows. Agrobacterial cells were collected from the overnight cultures in MG/L liquid medium, washed twice with AB-sucrose liquid medium, and then diluted to $OD_{600nm} = 0.1$ with AB-sucrose liquid medium. The cells were harvested after being cultured in AB-sucrose liquid medium at 28°C for the indicated time. The harvested cells were washed twice with PBS buffer and sonicated to near clarity. The cell lysate supernatants from these cell samples cultured for different times were collected by centrifugation ($12,000 \times g$ for 30 min) and adjusted to the same protein concentration. The red fluorescence intensities of these different supernatant samples were measured in triplicate using a Varian Cary Eclipse fluorescence spectrophotometer at an excitation wavelength of 553 nm and emission detection at 574 nm. Whole-cell lysate supernatant of wild-type C58 was used to eliminate the interference of background fluorescence.

ACKNOWLEDGMENTS

We thank R. Song (College of Veterinary Medicine, Yangzhou University) for help with confocal laser-microscopy observation.

LITERATURE CITED

- Abedrabbo, S., Castellon, J., Collins, K. D., Johnson, K. S., and Ottemann, K. M. 2017. Cooperation of two distinct coupling proteins creates chemosensory network connections. *Proc. Natl. Acad. Sci. U.S.A.* 114: 2970-2975.
- Adadevoh, J. S., Triolo, S., Ramsburg, C. A., and Ford, R. M. 2016. Chemotaxis increases the residence time of bacteria in granular media containing distributed contaminant sources. *Environ. Sci. Technol.* 50: 181-187.
- Adler, J. 1973. A method for measuring chemotaxis and use of the method to determine optimum conditions for chemotaxis by *Escherichia coli*. *J. Gen. Microbiol.* 74:77-91.
- Alexander, R. P., Lowenthal, A. C., Harshey, R. M., and Ottemann, K. M. 2010. CheV: CheW-like coupling proteins at the core of the chemotaxis signaling network. *Trends Microbiol.* 18:494-503.
- Alexandre, G. 2015. Chemotaxis control of transient cell aggregation. *J. Bacteriol.* 197:3230-3237.
- Alexandre, G., and Zhulin, I. B. 2003. Different evolutionary constraints on chemotaxis proteins CheW and CheY revealed by heterologous expression studies and protein sequence analysis. *J. Bacteriol.* 185: 544-552.
- Ashby, A. M., Watson, M. D., Loake, G. J., and Shaw, C. H. 1988. Ti plasmid-specified chemotaxis of *Agrobacterium tumefaciens* C58C¹ toward *vir*-inducing phenolic compounds and soluble factors from monocotyledonous and dicotyledonous plants. *J. Bacteriol.* 170: 4181-4187.

- Ashby, A. M., Watson, M. D., and Shaw, C. H. 1987. A Ti-plasmid determined function is responsible for chemotaxis of *Agrobacterium tumefaciens* towards the plant wound product acetosyringone. *FEMS Microbiol. Lett.* 41:189-192.
- Bi, S., and Lai, L. 2015. Bacterial chemoreceptors and chemoeffectors. *Cell. Mol. Life Sci.* 72:691-708.
- Boukhvalova, M., VanBruggen, R., and Stewart, R. C. 2002a. CheA kinase and chemoreceptor interaction surfaces on CheW. *J. Biol. Chem.* 277:23596-23603.
- Boukhvalova, M. S., Dahlquist, F. W., and Stewart, R. C. 2002b. CheW binding interactions with CheA and Tar. Importance for chemotaxis signaling in *Escherichia coli*. *J. Biol. Chem.* 277:22251-22259.
- Bourras, S., Rouxel, T., and Meyer, M. 2015. *Agrobacterium tumefaciens* gene transfer: How a plant pathogen hacks the nuclei of plant and nonplant organisms. *Phytopathology* 105:1288-1301.
- Briegleb, A., and Jensen, G. 2017. Progress and potential of electron cryotomography as illustrated by its application to bacterial chemoreceptor arrays. *Annu. Rev. Biophys.* 46:1-21.
- Browning, D. F., and Busby, S. J. 2016. Local and global regulation of transcription initiation in bacteria. *Nat. Rev. Microbiol.* 14:638-650.
- Butler, S. M., Nelson, E. J., Chowdhury, N., Faruque, S. M., Calderwood, S. B., and Camilli, A. 2006. Cholera stool bacteria repress chemotaxis to increase infectivity. *Mol. Microbiol.* 60:417-426.
- Cangelosi, G. A., Best, E. A., Martinetti, G., and Nester, E. W. 1991. Genetic analysis of *Agrobacterium*. *Methods Enzymol.* 204:384-397.
- Cardozo, M. J., Massazza, D. A., Parkinson, J. S., and Studdert, C. A. 2010. Disruption of chemoreceptor signalling arrays by high levels of CheW, the receptor-kinase coupling protein. *Mol. Microbiol.* 75:1171-1181.
- Chandran, V. 2013. Type IV secretion machinery: Molecular architecture and function. *Biochem. Soc. Trans.* 41:17-28.
- Charles, T. C., and Nester, E. W. 1993. A chromosomally encoded two-component sensory transduction system is required for virulence of *Agrobacterium tumefaciens*. *J. Bacteriol.* 175:6614-6625.
- Dogra, G., Purschke, F. G., Wagner, V., Haslbeck, M., Kriehuber, T., Hughes, J. G., Van Tassell, M. L., Gilbert, C., Niemeyer, M., Ray, W. K., Helm, R. F., and Scharf, B. E. 2012. *Sinorhizobium meliloti* CheA complexed with CheS exhibits enhanced binding to CheY1, resulting in accelerated CheY1 dephosphorylation. *J. Bacteriol.* 194:1075-1087.
- Erhardt, M. 2016. Strategies to block bacterial pathogenesis by interference with motility and chemotaxis. *Curr. Top. Microbiol. Immunol.* 398:185-205.
- Escobar, M. A., and Dandekar, A. M. 2003. *Agrobacterium tumefaciens* as an agent of disease. *Trends Plant Sci.* 8:380-386.
- Falke, J. J., and Piasta, K. N. 2014. Architecture and signal transduction mechanism of the bacterial chemosensory array: Progress, controversies, and challenges. *Curr. Opin. Struct. Biol.* 29:85-94.
- Gao, D., Bian, X., Guo, M., Wang, J., and Zhang, X. 2013. Identification and characterization of the biochemical function of *Agrobacterium* T-complex-recruiting protein Atu5117. *FEBS J.* 280:4865-4875.
- Gelvin, S. B. 2006. *Agrobacterium* virulence gene induction. *Methods Mol. Biol.* 343:77-84.
- Goodner, B., Hinkle, G., Gattung, S., Miller, N., Blanchard, M., Quorllo, B., Goldman, B. S., Cao, Y., Askenazi, M., Halling, C., Mullin, L., Houmiel, K., Gordon, J., Vaudin, M., Iartchouk, O., Epp, A., Liu, F., Wollam, C., Allinger, M., Doughty, D., Scott, C., Lappas, C., Markelz, B., Flanagan, C., Crowell, C., Gurson, J., Lomo, C., Sear, C., Strub, G., Cielo, C., and Slater, S. 2001. Genome sequence of the plant pathogen and biotechnology agent *Agrobacterium tumefaciens* C58. *Science* 294:2323-2328.
- Griswold, I. J., and Dahlquist, F. W. 2002. The dynamic behavior of CheW from *Thermotoga maritima* in solution, as determined by nuclear magnetic resonance: Implications for potential protein-protein interaction sites. *Biophys. Chem.* 101-102:359-373.
- Guo, M., Bian, X., Wu, X., and Wu, M. 2011. *Agrobacterium*-mediated genetic transformation: History and progress. Pages 3-28 in: *Genetic Transformation*. M. Alvarez, ed. InTech, Rijeka, Croatia.
- Guo, M., Gao, D., and Jin, Y. 2009a. Progress in the formation and transfer of *Agrobacterium* T-complex. *Prog. Biochem. Biophys.* 36:1408-1414.
- Guo, M., Hou, Q., Hew, C. L., and Pan, S. Q. 2007a. *Agrobacterium* VirD2-binding protein is involved in tumorigenesis and redundantly encoded in conjugative transfer gene clusters. *Mol. Plant-Microbe Interact.* 20:1201-1212.
- Guo, M., Huang, Z., and Yang, J. 2017. Is there any crosstalk between the chemotaxis and virulence induction signaling in *Agrobacterium tumefaciens*? *Biotechnol. Adv.* 35:505-511.
- Guo, M., Jin, S., Sun, D., Hew, C. L., and Pan, S. Q. 2007b. Recruitment of conjugative DNA transfer substrate to *Agrobacterium* type IV secretion apparatus. *Proc. Natl. Acad. Sci. U.S.A.* 104:20019-20024.
- Guo, M., Zhu, Q., and Gao, D. 2009b. Development and optimization of method for generating unmarked *A. tumefaciens* mutants. *Prog. Biochem. Biophys.* 36:556-565.
- Harighi, B. 2008. Role of CheY1 and CheY2 in the chemotaxis of *A. tumefaciens* toward acetosyringone. *Curr. Microbiol.* 56:547-552.
- Harighi, B. 2009. Genetic evidence for CheB- and CheR-dependent chemotaxis system in *A. tumefaciens* toward acetosyringone. *Microbiol. Res.* 164:634-641.
- Hawes, M. C., and Smith, L. Y. 1989. Requirement for chemotaxis in pathogenicity of *Agrobacterium tumefaciens* on roots of soil-grown pea plants. *J. Bacteriol.* 171:5668-5671.
- Hawes, M. C., Smith, L. Y., and Howarth, A. J. 1988. *Agrobacterium tumefaciens* mutants deficient in chemotaxis to root exudates. *Mol. Plant-Microbe Interact.* 1:182-186.
- He, K., and Bauer, C. E. 2014. Chemosensory signaling systems that control bacterial survival. *Trends Microbiol.* 22:389-398.
- Hess, J. F., Oosawa, K., Kaplan, N., and Simon, M. I. 1988. Phosphorylation of three proteins in the signaling pathway of bacterial chemotaxis. *Cell* 53:79-87.
- Jones, C. W., and Armitage, J. P. 2015. Positioning of bacterial chemoreceptors. *Trends Microbiol.* 23:247-256.
- Kim, H., and Farrand, S. K. 1998. Opine catabolic loci from *Agrobacterium* plasmids confer chemotaxis to their cognate substrates. *Mol. Plant-Microbe Interact.* 11:131-143.
- Krell, T., Lacal, J., Reyes-Darias, J. A., Jimenez-Sanchez, C., Sungthong, R., and Ortega-Calvo, J. J. 2013. Bioavailability of pollutants and chemotaxis. *Curr. Opin. Biotechnol.* 24:451-456.
- Kudryasheva, N. S., and Tarasova, A. S. 2015. Pollutant toxicity and detoxification by humic substances: Mechanisms and quantitative assessment via luminescent biomonitoring. *Environ. Sci. Pollut. Res. Int.* 22:155-167.
- Lang, J., and Faure, D. 2014. Functions and regulation of quorum-sensing in *Agrobacterium tumefaciens*. *Front. Plant Sci.* 5:14.
- Li, Y., Hu, Y., Fu, W., Xia, B., and Jin, C. 2007. Solution structure of the bacterial chemotaxis adaptor protein CheW from *Escherichia coli*. *Biochem. Biophys. Res. Commun.* 360:863-867.
- Li, Z.-H., Dong, K., Sun, J.-C., Yuan, J.-P., Hu, B.-Y., Liu, J.-X., Zhao, G.-P., and Guo, X.-K. 2006. Characterization of *cheW* genes of *Leptospira interrogans* and their effects in *Escherichia coli*. *Acta Biochim. Biophys. Sin. (Shanghai)* 38:79-88.
- Liu, J. D., and Parkinson, J. S. 1989. Role of CheW protein in coupling membrane receptors to the intracellular signaling system of bacterial chemotaxis. *Proc. Natl. Acad. Sci. U.S.A.* 86:8703-8707.
- Liu, J. D., and Parkinson, J. S. 1991. Genetic evidence for interaction between the CheW and Tsr proteins during chemoreceptor signaling by *Escherichia coli*. *J. Bacteriol.* 173:4941-4951.
- Loake, G. J., Ashby, A. M., and Shaw, C. H. 1988. Attraction of *Agrobacterium tumefaciens* C58C¹ towards sugars involves a highly sensitive chemotaxis system. *Microbiology* 134:1427-1432.
- Low, H. H., Gubellini, F., Rivera-Calzada, A., Braun, N., Connery, S., Dujeancourt, A., Lu, F., Redzej, A., Fronzes, R., Orlova, E. V., and Waksman, G. 2014. Structure of a type IV secretion system. *Nature* 508:550-553.
- Lowenthal, A. C., Simon, C., Fair, A. S., Mehmood, K., Terry, K., Anastasia, S., and Ottemann, K. M. 2009. A fixed-time diffusion analysis method determines that the three *cheV* genes of *Helicobacter pylori* differentially affect motility. *Microbiology* 155:1181-1191.
- Mangwani, N., Kumari, S., and Das, S. 2016. Bacterial biofilms and quorum sensing: Fidelity in bioremediation technology. *Biotechnol. Genet. Eng. Rev.* 32:43-73.
- Martin, A. C., Wadhams, G. H., and Armitage, J. P. 2001. The roles of the multiple CheW and CheA homologues in chemotaxis and in chemoreceptor localization in *Rhodobacter sphaeroides*. *Mol. Microbiol.* 40:1261-1272.
- Matveeva, T. V., and Lutova, L. A. 2014. Horizontal gene transfer from *Agrobacterium* to plants. *Front. Plant Sci.* 5:326.
- McCullen, C. A., and Binns, A. N. 2006. *Agrobacterium tumefaciens* and plant cell interactions and activities required for interkingdom macromolecular transfer. *Annu. Rev. Cell Dev. Biol.* 22:101-127.
- Merritt, P. M., Danhorn, T., and Fuqua, C. 2007. Motility and chemotaxis in *Agrobacterium tumefaciens* surface attachment and biofilm formation. *J. Bacteriol.* 189:8005-8014.
- Minchin, S. D., and Busby, S. J. 2009. Analysis of mechanisms of activation and repression at bacterial promoters. *Methods* 47:6-12.
- Montaño, M., Gutleb, A. C., and Murk, A. J. 2013. Persistent toxic burdens of halogenated phenolic compounds in humans and wildlife. *Environ. Sci. Technol.* 47:6071-6081.
- Nester, E. W. 2015. *Agrobacterium*: Nature's genetic engineer. *Front. Plant Sci.* 5:730.

- Nishiyama, S., Garzón, A., and Parkinson, J. S. 2014. Mutational analysis of the PI phosphorylation domain in *Escherichia coli* CheA, the signaling kinase for chemotaxis. *J. Bacteriol.* 196:257-264.
- Ortega, D. R., and Zhulin, I. B. 2016. Evolutionary genomics suggests that CheV is an additional adaptor for accommodating specific chemoreceptors within the chemotaxis signaling complex. *PLOS Comput. Biol.* 12:e1004723.
- Parke, D., Ornston, L. N., and Nester, E. W. 1987. Chemotaxis to plant phenolic inducers of virulence genes is constitutively expressed in the absence of the Ti plasmid in *Agrobacterium tumefaciens*. *J. Bacteriol.* 169:5336-5338.
- Parkinson, J. S., Hazelbauer, G. L., and Falke, J. J. 2015. Signaling and sensory adaptation in *Escherichia coli* chemoreceptors: 2015 Update. *Trends Microbiol.* 23:257-266.
- Parkinson, J. S., and Houts, S. E. 1982. Isolation and behavior of *Escherichia coli* deletion mutants lacking chemotaxis functions. *J. Bacteriol.* 151:106-113.
- Piñas, G. E., Frank, V., Vaknin, A., and Parkinson, J. S. 2016. The source of high signal cooperativity in bacterial chemosensory arrays. *Proc. Natl. Acad. Sci. U.S.A.* 113:3335-3340.
- Pittman, M. S., Goodwin, M., and Kelly, D. J. 2001. Chemotaxis in the human gastric pathogen *Helicobacter pylori*: Different roles for CheW and the three CheV paralogues, and evidence for CheV2 phosphorylation. *Microbiology* 147:2493-2504.
- Pitzschke, A. 2013. *Agrobacterium* infection and plant defense-transformation success hangs by a thread. *Front. Plant Sci.* 4:519.
- Porter, S. L., Wadhams, G. H., and Armitage, J. P. 2011. Signal processing in complex chemotaxis pathways. *Nat. Rev. Microbiol.* 9:153-165.
- Rosario, M. M., Fredrick, K. L., Ordal, G. W., and Helmann, J. D. 1994. Chemotaxis in *Bacillus subtilis* requires either of two functionally redundant CheW homologs. *J. Bacteriol.* 176:2736-2739.
- Salah Ud-Din, A. I. M., and Roujeinikova, A. 2017. Methyl-accepting chemotaxis proteins: A core sensing element in prokaryotes and archaea. *Cell. Mol. Life. Sci.* 74:3293-3303.
- Sambrook, J. F., Fritsch, E. F., and Maniatis, T. 1989. *Molecular Cloning: A Laboratory Manual*, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
- Sampedro, I., Parales, R. E., Krell, T., and Hill, J. E. 2015. *Pseudomonas* chemotaxis. *FEMS Microbiol. Rev.* 39:17-46.
- Scharf, B. E., Hynes, M. F., and Alexandre, G. M. 2016. Chemotaxis signaling systems in model beneficial plant-bacteria associations. *Plant Mol. Biol.* 90:549-559.
- Shaw, C. H. 1995. *Agrobacterium tumefaciens* chemotaxis protocols. *Methods Mol. Biol.* 44:29-36.
- Sourjik, V., and Berg, H. C. 2000. Localization of components of the chemotaxis machinery of *Escherichia coli* using fluorescent protein fusions. *Mol. Microbiol.* 37:740-751.
- Sourjik, V., and Wingreen, N. S. 2012. Responding to chemical gradients: Bacterial chemotaxis. *Curr. Opin. Cell Biol.* 24:262-268.
- Springer, M. S., Goy, M. F., and Adler, J. 1977. Sensory transduction in *Escherichia coli*: Two complementary pathways of information processing that involve methylated proteins. *Proc. Natl. Acad. Sci. U.S.A.* 74:3312-3316.
- Stachel, S. E., Messens, E., Van Montagu, M., and Zambryski, P. 1985. Identification of the signal molecules produced by wounded plant cells that activate T-DNA transfer in *Agrobacterium tumefaciens*. *Nature* 318:624-629.
- Su, S., Stephens, B. B., Alexandre, G., and Farrand, S. K. 2006. Lon protease of the α -proteobacterium *Agrobacterium tumefaciens* is required for normal growth, cellular morphology and full virulence. *Microbiology* 152:1197-1207.
- Subramoni, S., Nathoo, N., Klimov, E., and Yuan, Z. C. 2014. *Agrobacterium tumefaciens* responses to plant-derived signaling molecules. *Front. Plant Sci.* 5:322.
- Wood, D. W., Setubal, J. C., Kaul, R., Monks, D. E., Kitajima, J. P., Okura, V. K., Zhou, Y., Chen, L., Wood, G. E., Almeida, N. F., Jr., Woo, L., Chen, Y., Paulsen, I. T., Eisen, J. A., Karp, P. D., Bovee, D., Sr., Chapman, P., Clendenning, J., Deatherage, G., Gillet, W., Grant, C., Kutayavin, T., Levy, R., Li, M. J., McClelland, E., Palmieri, A., Raymond, C., Rouse, G., Saenphimmachak, C., Wu, Z., Romero, P., Gordon, D., Zhang, S., Yoo, H., Tao, Y., Biddle, P., Jung, M., Krespan, W., Perry, M., Gordon-Kamm, B., Liao, L., Kim, S., Hendrick, C., Zhao, Z. Y., Dolan, M., Chumley, F., Tingey, S. V., Tomb, J. F., Gordon, M. P., Olson, M. V., and Nester, E. W. 2001. The genome of the natural genetic engineer *Agrobacterium tumefaciens* C58. *Science* 294:2317-2323.
- Wright, E. L., Deakin, W. J., and Shaw, C. H. 1998. A chemotaxis cluster from *Agrobacterium tumefaciens*. *Gene* 220:83-89.
- Yang, J., Wu, M., Zhang, X., Guo, M., and Huang, Z. 2015. Expression of *Agrobacterium* homolog genes encoding T-complex recruiting protein under virulence induction conditions. *Front. Microbiol.* 6:1379.
- Yu, M., Zuo, J., Gu, H., Guo, M., and Yin, Y. 2015. Domain function dissection and catalytic properties of *Listeria monocytogenes* p60 protein with bacteriolytic activity. *Appl. Microbiol. Biotechnol.* 99:10527-10537.
- Zhang, K., Liu, J., Tu, Y., Xu, H., Charon, N. W., and Li, C. 2012. Two CheW coupling proteins are essential in a chemosensory pathway of *Borrelia burgdorferi*. *Mol. Microbiol.* 85:782-794.
- Zhao, J., and Parkinson, J. S. 2006a. Cysteine-scanning analysis of the chemoreceptor-coupling domain of the *Escherichia coli* chemotaxis signaling kinase CheA. *J. Bacteriol.* 188:4321-4330.
- Zhao, J., and Parkinson, J. S. 2006b. Mutational analysis of the chemoreceptor-coupling domain of the *Escherichia coli* chemotaxis signaling kinase CheA. *J. Bacteriol.* 188:3299-3307.