University of California

Santa Cruz

CheV1 Leads the Organization of Chemotaxis Signal Transduction Proteins in *Helicobacter pylori*

A thesis submitted in partial satisfaction
of the requirements for the degree of

MASTER OF SCIENCE

in

MICROBIOLOGY AND ENVIRONMENTAL TOXICOLOGY

by

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June 2013

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ABSTRACT

Helicobacter pylori translates environmental cues into a swimming response using its chemotactic signaling system. Generally, chemotactic signaling in *H. pylori* follows the enteric paradigm. The *H. pylori* system, however, has three additional proteins called CheVs that are hybrid proteins combining a CheW domain with a response receiver (REC) domain. Studies on *H. pylori* have shown that each CheV participates in wild-type chemotaxis although the exact role of each protein in the chemotactic signaling system is unclear. The goal of this study is to better understand the function of CheVs in the pathway, by specifically testing spatial localization of the CheV proteins and whether they prefer specific chemoreceptors. We carried out subcelluar fractionation followed by western analysis to determine whether any of the *H. pylori* CheV proteins prefer the TlpA or TlpB chemoreceptors. Additionally, immunofluorescence was applied to observe the localization of each CheV in wild-type *H. pylori*, in isogenic strains having only TlpA or TlpB, and in isogenic strains lacking one CheV or CheW. We found that all three CheV proteins are detected in membrane fractions with either TlpA or TlpB, and surprisingly, even in the absence of all receptors. This outcome suggested that CheV proteins associate with the membrane independently of the chemoreceptors. In wild type, we found that the CheVs are primarily localized at the cell poles. In contrast, strains lacking

chemoreceptors had CheV proteins diffused throughout the cytoplasm. Strains bearing a single chemoreceptor had CheVs both at the poles and diffused within the cell. In the absence of CheV1, both CheV2 and CheV3 where diffused; on the contrary, loss of either CheV2 or CheV3 did not affect the localization of the remaining CheV proteins. Immunofluorescence studies also suggested that both CheV1 and CheW are involved in localizing the chemoreceptors to the cell pole. These results suggest that either TlpA or TlpB can guide the localization of CheVs to the cell pole. In a cell with only a single chemoreceptor, the quantity of chemoreceptor complex formed by TlpA or TlpB is altered and appears to affect CheV localization. These studies suggest that the *H. pylori* CheV proteins do not prefer TlpA or TlpB, and instead interact with all chemoreceptors equally. Unlike the other CheV proteins, CheV1 showed a significant effect on the polar localization of chemoreceptors, and concomitantly, CheV2 and CheV3. CheW similarly was required for polar chemoreceptors. These studies thus show that the *H. pylori* CheV1 and CheW proteins have a major role in the spatial localization of chemotaxis transduction proteins.

ACKKNOWLEDGEMENTS

This work would not have been possible without the guidance from several individuals. First and foremost, I thank my mentor and advisor, professor Karen M. Ottemann, who has supported me throughout my investigations with her guidance, motivation and professional advice. I attribute my development as a scientist to her effort and encouragement. Thank you for being an excellent role model and for your dedication to a life of scientific pursuit. You have touched the lives of many with your example, words of wisdom, and unwavering support.

While investigating *Helicobacter pylori*, I had the pleasure of working with an intriguing research group. I thank my fellow present and past Ottemann lab members: Kieran Collins, William E. Sause, Susan Williams, Annah S. Rolig, Pam Lertsethtakarn, and Lisa M. Collison for helpful advice and shared laughs.

Lastly my biggest supporters Catalina and Vidal Castellón, Mom and Dad, thank you for the love and support throughout these years. Gracias por su apoyo en mis tiempos de fracoso y en todos mis éxitos. Sin su comprensión, guía, y sin el ejemplo que siempre me han dado de ser perseverante, paciente y honesto no hubiera logrado mis éxitos. Gracias por ser un modelo ejemplar para mí, los amo. Este tesis es dedicado a ustedes.

Introduction

Helicobacter pylori, a human pathogen that colonizes the stomach and causes gastric ulcers and potentially stomach cancer, requires motility and chemotaxis to establish initial infection. Several bacterial species rely on chemotaxis to move toward favorable chemical gradients or away from unfavorable ones, with the goal to always find beneficial and plentiful nutrients (Berg and Brown 1972; Larsen, Reader et al. 1974; Garrity and Ordal 1997; Wadhams and Armitage 2004). The chemosensory machinery is a critical factor in interpreting beneficial chemical gradients (Sourjik 2004).

Extensive studies in enteric bacteria *Escherichia coli* and *Salmonella enterica* serovar Typhimurium helped to tease apart the mechanism(s) of chemotaxis and given us a better understanding of how bacteria interpret chemical cues. The enteric paradigm utilizes integral membrane receptors, also called methyl-accepting chemotactic proteins (MCPs), histidine kinase sensors, coupling proteins, response regulators (RR) that contain REC or receiver domains, and enzymes (methyl transferase, methyl esterase, phosphatase). All components cooperatively work to form a signal transduction network that allows bacteria to interpret spatial gradients of chemoeffectors as they explore the ever-changing environment.

The first components of this pathway are chemoreceptors, which sense chemical gradients in the environment. Upon binding of chemoeffector, the ligand-binding information is relayed to a histidine kinase sensor, CheA, the second component of the signaling cascade and part of a two-component regulatory system

present in several signaling pathways in bacteria. Moreover, the coupling protein CheW binds to both MCP and CheA to allow a physical connection between them. CheA is a kinase that transfers a phophoryl group to target REC domains (Gegner, Graham et al. 1992). Therefore, a phosphorylated CheA is active and ready to transfer the signal by donating a phosphoryl group to the response regulator, CheY. Then phosphorylated CheY adopts a form that enables the protein to interact with the flagellar apparatus.

Several microbial species follow the enteric chemotactic paradigm, however some bacteria have additional proteins involved in the signaling pathway. One such additional protein is a hybrid protein named CheV, which combines a CheW domain with a REC domain (Fredrick and Helmann 1994; Pittman, Goodwin et al. 2001; Lowenthal, Simon et al. 2009). CheV proteins are found in different species of bacteria and these can have a single CheV or up to three CheVs (Alexander, Lowenthal et al. 2010). *H. pylori*, for example, has three CheVs (CheV1, CheV2, CheV3) and all have been shown to regulate chemotaxis (Lowenthal, Simon et al. 2009). A recent study suggested that significant charge and residue conservation is present in the coupling domain of CheVs, therefore all three proteins possibly interact with chemoreceptors and CheA (Lowenthal, Simon et al. 2009). Moreover, a soft-agar analysis found that a null *cheV1* strain had a severe decrease in soft-agar migration, which is consistent to a previous study, while null CheV2 and null CheV3 displayed a subtle but significant decrease and increase in migration, respectively (Pittman, Goodwin et al. 2001; Lowenthal, Simon et al. 2009). To better assess the

swimming behavior of strains mutant for CheVs, a fixed-time diffusion measurement was applied and results showed that CheV1 and CheV2 mutants were smooth swimming, similar to the CheY mutant, but the CheV3 mutant had a different behavior, consisting of increased changes of direction (Lowenthal, Simon et al. 2009). Importantly all three CheVs had an effect on flagellar rotation, providing the first evidence that all three do act in the chemotaxis pathway (Lowenthal, Simon et al. 2009). *H. pylori* also possesses a protein called ChePep, which was also shown to regulate chemotaxis in *H. pylori* (Howitt, Lee et al. 2011). ChePep was shown to localize at the flagellar pole of *H. pylori* and under a high pH gradient it mediates the response to acid exposure. Cleary these additional proteins affect the chemosensory pathway and their intracellular localization is important.

Previous studies in *E. coli* showed chemoreceptors arrange into arrays and localize at the cell poles with CheW and CheA, the coupler and histidine kinase, respectively (Alley, Maddock et al. 1992; Maddock and Shapiro 1993; Briegel, Ortega et al. 2009). The kinase, coupler, and chemoreceptor form a "ternary" complex that allows the bacterium to interpret chemical cues in the surrounding environment and respond by moving toward favorable chemical gradients (Bray, Levin et al. 1998; Sourjik 2004; Wadhams and Armitage 2004). How these chemotaxis signaling complexes localize to the pole is not known. One protein that plays a role in this has been identified in *Vibrio cholerae.* Specifically, the absence of HubP resulted in a negative affect on chemotaxis and loss of the polar assembly of the chemoreceptor arrays (Yamaichi, Bruckner et al. 2012). Major components of the

chemosensory machinery have been shown to localize at the cell pole in many non-pathogenic bacterial species, however, the intracellular localization of the components in the chemosensory pathway has been less explored in pathogenic bacteria. Therefore, the focus of this work is threefold: (1) to investigate the spatial localization of the CheV proteins; (2) determine whether they prefer a specific chemoreceptor; (3) observe the role of CheV proteins to direct the polar localization of one another and the chemoreceptors.

Experimental Procedures

H. pylori strains and culture conditions

For liquid culture, *Helicobacter pylori* strain mG27 (wild-type) and isogenic mutant strains with a single chemoreceptor, no chemoreceptors, and single mutants of *cheV* were grown in brucella broth (Difco) with 10% heat inactivated fetal bovine serum (Gibco) (BB10) at 37° C under 10% CO_2, 5% O_2, and 83% N_2. All strains are listed in Table 1. For solid culture, *H. pylori* strains was grown on Columbia blood agar with 5% defibrinated horse blood and 50 μg/mL cyclohexamide (CHBA) plates with minimal passage and incubated at the same temperature and conditions as stated for liquid cultures. When appropriate, antibiotics were included at the following concentrations: 5 – 10 μg/mL chloramphenicol and 15 μg/mL kanamycin.

Cellular fractionation

H. pylori strain mG27 and mutant strains were cultured on Colombia horse blood agar plates with minimal passages. Cells were resuspended from the plate into lysis buffer (50mM Tris-HCl, 10% glycerol, 1mM AEBSF, 10mM DTT), chilled on

ice and sonicated in 30 second bursts until the lysate appeared clear. Unlysed bacterial cells were pelleted at 4,000g at 4°C for 10 minutes and the supernatant collected. To separate membrane from soluble fractions the supernatant was spun by ultracentrifugation at 100,000g for 40 minutes at 4°C. A high salt buffer (lysis buffer plus 2M KCl) was used to vigorously wash the membrane pellet 3X, followed by ultracentrifugation as above. Membranes were resuspended in lysis buffer and stored at -20°C. Membrane fractions were normalized by the use of Bradford assay, with bovine serum albumin (BSA) as a standard.

Immunoblotting

Membrane samples, 10µg per lane, were separated by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Separated proteins were transferred to a polyvinylidene diflouride membrane (PVDF) then blotted with anti-CheV1, anti-CheV2, or anti-CheV3 polyclonal antibodies (see below) at a dilution of 1:54. The blot was stripped with a western blot stripping buffer (GM Biosciences) and reprobed for repeated analysis. For visualization, a secondary antibody chicken anti-rabbit conjugate to horseradish peroxidase (Santa Cruz Biotech) at a dilution of 1:1000 was used, followed by incubation with luminol.

For generation of antibodies, CheV1, CheV2, or CheV3 proteins were overexpressed in *E. coli* BL21. Cultures were grown in Luria-Bertani (LB) medium or 2XYT plus ampicillin to OD_{600} 0.5-0.8 and induced with 0.1-1 mM of IPTG for 4-16 hours at 37°C. Cells were harvested by centrifugation, lysed by sonication and then centrifuged at 206,020g for 45 minutes to remove membranes and unlysed cells. The

supernatant was applied to a GST Prep 16/10 column (GE Healthcare) for purification of GST-tagged proteins. Purified proteins were cleaved from GST using Precission Protease (GE) and sent for antibodies generation in rabbits: CheV1 to Animal Pharm Services, Inc. in Healdsburg, CA and CheV2 and CheV3 to Cocalico Biologicals, Inc. in Reamstown, PA. The α-GST-TlpA22 were generated as described previously (Williams, Chen et al. 2007; Lowenthal, Hill et al. 2009).

Preabsorption of Antibodies

Mutant strains of *H. pylori*, G27 Δ*cheV1::cat*, G27 Δ*cheV2::cat*, and G27 Δ*cheV3::cat* were used for preabsorption of anti-CheV1, anti-CheV2, and anti-CheV3, respectively. The mutant strains with a single or no chemoreceptors present were used for preabsorption of anti-TlpA22. For each strain, cells were grown on three CHBA plates. Bacterial cells were collected into in 2mL 1X PBS and pelleted by centrifugation at 7,000 RPM for 10 minutes (Eppendorf F45-24-11 rotor in Eppendorf 5415 D centrifuge). The pellet was resuspended in 2ml PLP (75mM $NaPO_4$, pH 7.4, 2.5mM NaCl, 2% paraformaldehyde in 1X PBS) and incubated for 10 minutes at room temperature. Cells were centrifuged as previously mentioned and the pellet resuspended in 2ml 1X PBS. This step was done three times. To pre-absorb against proteins of interest, 1ml of buffer 2 (3% BSA, 1% Saponin, 0.1% triton X-100, 0.02% sodium azide in 1X PBS) was used to resuspend pellet, followed by incubation for 10 minutes at room temperature. Cells were centrifuged as above and supernatant removed. The pellet was resuspended in 700µl of buffer 2 plus antibody diluted to 1:100 and rotated overnight at 4ºC. Cells were centrifuged at 10,000 rpm for 10

minutes (eppendorf F45-24-11 rotor in eppendorf 5415 D centrifuge) and supernatant was centrifuged once more to remove debri. The methodology for immunofluorescence and pre-absorption of antibodies were provided by Michael Howitt (Stanford, personal communication).

Immunofluorescence Microscopy

Batch cultures of *H. pylori* were grown in BB10 in microaerobic conditions with shaking at 37°C for approximately 15 hours, to an optical density at 600nm (OD_{600}) for each strain used for this study. Logarithmic growing cells were visually inspected for motility with phase contrast microscopy prior to use. Motile bacteria were spotted and immobilized onto poly-L-lysine coated microscope slides (Fischer, Jacobson et al. 2008). Cells were fixed and permeabilized as previously described (Pentecost, Otto et al. 2006; Howitt, Lee et al. 2011). Briefly, cells were fixed with PLP at room temperature for 10 minutes, followed by washing three times with 1X phosphate-buffer saline (PBS). The permeabilization of cells was carried out with permeabilization buffer (3% BSA and 0.1% Triton X-100 in 1X PBS) at room temperature for 10 minutes. Pre-absorbed primary antibodies specific to chemoreceptors, CheV1, CheV2, CheV3 and chicken anti-*H. pylori* (GENTAUR) were incubated at, 1:200, 1:50 and 1:500, respectively, for 30 minutes at room temperature without movement. This step was followed by washing 3 times with permeabilization buffer before the addition of secondary antibodies. Alexa Fluor conjugated antibodies were used for secondary detection (Invitrogen Molecular Probes™). Goat anti-chicken conjugated to Alexa Flour® 594 (594 channel-red) or

goat anti-rabbit conjugated to Alexa Flour® 488 (488 channel-green) were incubated at 1:500 and 1:300, respectively, for 30 minutes at room temperature without movement and light exposure. Cells were washed as previously stated and a drop of Vectashield® was added prior to placing the cover slip.

Fluorescent bacterial cells were imaged using a Nikon Eclipse E600 fluorescent microscope equipped with a Plan Flour 100X oil immersion objective lens (Nikon). To view and capture images from Alexa Flour® 594 and Alexa Flour® 488, the Texas Red® (Chroma) and FITC/GFP (Chroma) filter cube were used, respectively. Images were captured with SPOT insight fire wire 4 mega camera and the SPOT software version 4.7 (Diagnostic instruments, inc.). Fluorescent images were assembled in Adobe® Photoshop® CS2 version 9.0.2 (Adobe®). The image from one fluor was copied and pasted to the second image of the second fluor and the difference blend mode was used to merge both the images, followed by adjustment of brightness between 0 to 60- value. The scale bars on fluorecent images are 2 μm.

The number of immunofluorecent bacteria was observed in different random fields using the 100X objective. To determine the localization of a protein the following was applied. A protein exclusively observed at the pole or the tip of the bacterial cell with no distinct signal within the cell was scored as “polar” localization. Bacterial cells having a signal at the tip of the cell and throughout the cell were scored as “polar and diffuse”. Although not seen, we would have scored any cells without a strong polar signal as “diffuse”. Multiple of three scores were performed and localization scores were added and divided by total cell number and multiplied by

100 to get the percent of protein localization of either "polar" or "polar and diffuse" localization. The quantification was performed on at least 150 cells per experiment.

Results

CheVs (CheV1, CheV2, CheV3) interact non-specifically with the membrane

To test whether CheVs have a preference for any specific chemoreceptor, we isolated cell membranes from *H. pylori* strains that contained only TlpA or TlpB chemoreceptors, or for controls, *H. pylori* strains that lacked all chemoreceptors (Table 1). These membranes were then incubated with polyclonal antibodies specific for CheV1, CheV2, or CheV3. Control experiments showed that the antibodies were specific to each CheV (Fig. 1). We detected each CheV protein (V1, V2, and V3) in membrane fractions with TlpA or TlpB, implying that all three CheV proteins interact with TlpA or TlpB (Fig. 1). Surprisingly the three CheV proteins were also detected in membrane fractions missing all chemoreceptors (Fig. 1). This finding suggested that either the CheV proteins interact non-specifically with membranes, or that they interact with another protein such as CheA, which a previous study has shown to associate with membrane fractions (Garrity and Ordal 1997). These results were consistent with the idea that CheVs do not prefer a particular chemoreceptor, and furthermore that cellular fractionation was not a good method to determine chemoreceptor preference. We thus turned to a different methodology, immunofluorescence.

Table 1. *Helicobacter pylori* strains used in this study

Strains	Genotype	Source
H. pylori mG27	WT	Lab Collection
G27 Δ*cheV1*	Δ*cheV1::cat*	Lab Collection
G27 Δ*cheV2*	Δ*cheV2::cat*	Lab Collection
G27 Δ*cheV3*	Δ*cheV3::cat*	Lab Collection
mG27 Δ*cheADC*	Δ*tlpA,* Δ*tlpD::cat,* Δ*tlpC::aphA3*	Lab Collection
mG27 Δ*tlpBCD*	Δ*tlpB,* Δ*tlpC::aphA3,* Δ*tlpD::cat*	Lab Collection
mG27 Δ*tlpABCD*	Δ*tlpA,* Δ*tlpD::cat,* Δ*tlpB,* Δ*tlpC::aphA3,*	Lab Collection

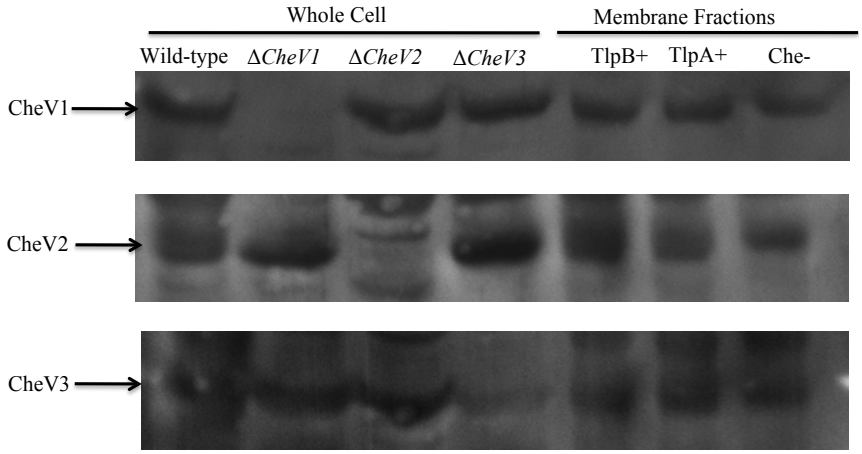

Figure 1. CheVs (Chev1, CheV2, CheV3) interact non-specifically with the membrane. The detection of CheVs (CheV1, CheV2, CheV3) in the presence of a single chemoreceptor (TlpB+ or TlpA+) and in the absence of all chemoreceptors (Che-) was determined by immunoblotting with native antibody specific to each CheV. Blot was stripped prior to probing with CheV2 or CheV3.

Immunofluorescence microscopy demonstrates that single chemoreceptors, TlpA or TlpB, guide partial polar localization of each CheV

Immunofluorescence was applied to see the spatial localization of all three CheV proteins in cells expressing CheV from their native position and at wild-type levels. This approach showed that all three CheV proteins localize to the cell pole in wild-type *H. pylori* (Fig. 2a). Moreover, greater than 90% of cells show all three CheV proteins primarily localized to the cell pole (Fig. 2b). This localization is similar to that seen for other chemotaxis proteins including CheA and CheY (Lertsethtakarn 2011), suggesting that the three CheV proteins form part of the chemoreceptor complex. To further explore the role of chemoreceptors in guiding CheVs to the cell pole, a strain missing all chemoreceptors was probed for each CheV protein. In the absence of all chemoreceptors, each CheV protein dispersed throughout the cell (Fig. 2c). This result suggests that TlpA, TlpB and/or TlpD chemoreceptor populations are necessary for wild-type CheV polar localization, and suggest that CheVs localize with the chemoreceptor-CheW-CheA complex at the poles. We then tested whether a single chemoreceptor was sufficient to sequester each CheV to the cell pole. Isogenic mutants having a single chemoreceptor (TlpA or TlpB) were used to visualize the localization of the three CheVs. The immunofluorescence results showed CheVs localize to the pole and distribute within the cell in the presence of a single chemoreceptor, TlpA or TlpB (Fig. 2d). When TlpA was present, between 90% - 92% of cells had CheV3 and CheV1 both at the poles and diffused (Fig. 2e). 87% of cells showed CheV2 localization similar to the

two CheVs (Fig. 2e). In the presence of TlpB, 91% of cells had CheV1 at the pole and diffused, 85% of cells showed CheV2 with a similar localization, and 92% of cells showed CheV3 localized at the pole and diffused as well (Fig. 2f). Thus, these results showed that neither a single TlpA or TlpB population is able to completely localize the three CheV proteins to the cell pole, however each can provide modest polar localization. Our findings furthermore suggest that both types of chemoreceptors—TlpA and TlpB—are required for the distinct polar localization of the three CheV proteins observed in wild type.

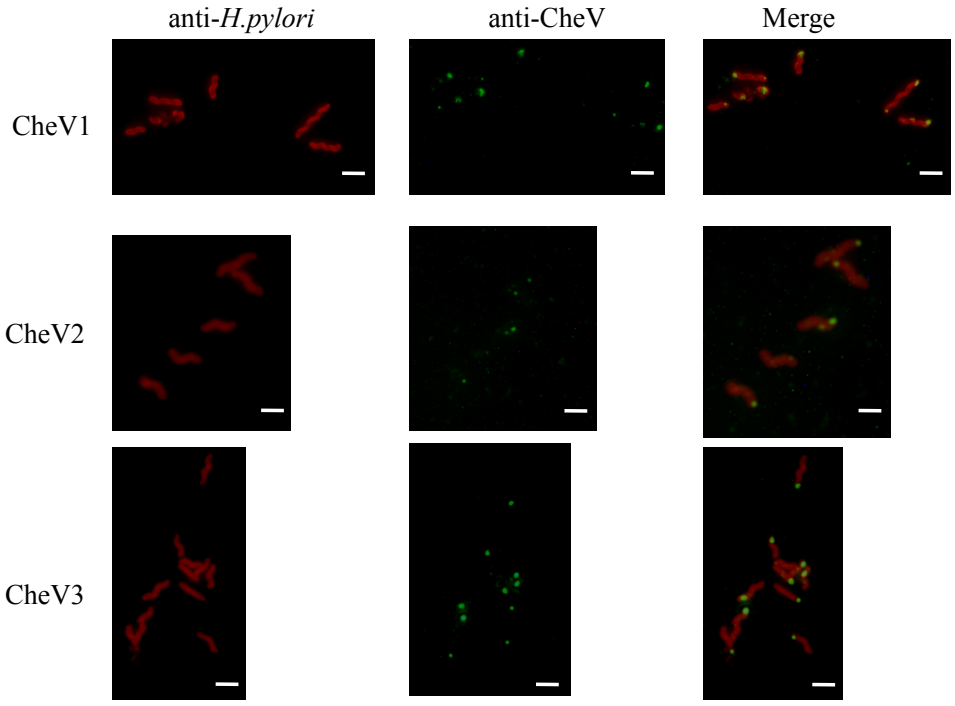

Figure 2a. In wild type, the three CheV proteins localize to the cell pole. Immunofluorescence microscopy shows that CheVs have a distinct polar localization in exponentially growing bacterial cells. Chicken anti-*H. pylori* Alexa red recognizes *H. pylori*, while anti-rabbit Alexa green recognizes CheVs. Merge images combines both images to produce a red color for *H. pylori* and a green/yellow color for CheVs.

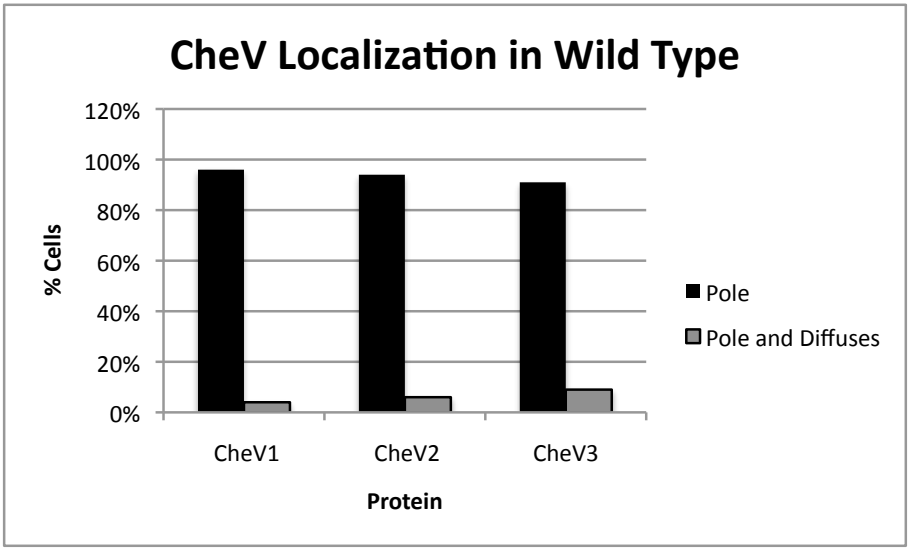

Figure 2b. Single-cell quantification of subcellular immunofluorescence localization of CheV1 CheV2 and CheV3. In wild-type, 96% of cells showed CheV1at the pole, 94% of cells showed CheV2 at the pole, and 91% of cells showed CheV 3 at the pole. The cell distribution data presented in the graph were obtained from cells represented in figure2a treated with specific Alexa Fluor and visualized by fluorescence microscopy. The quantification was performed on at least 150 cells per experiment.

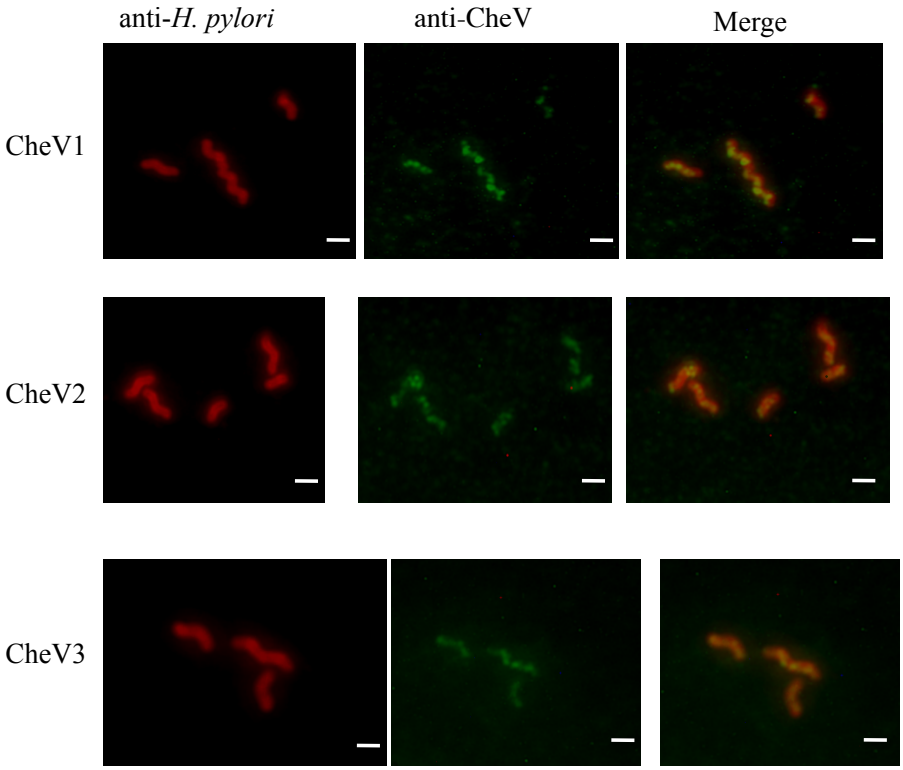

Figure 2c. In the absence of all chemoreceptors, CheV1 CheV2 and CheV3 are diffused throughout the bacterial cell. Chicken anti-*H. pylori* Alexa red recognizes *H. pylori*, while anti-rabbit Alexa green recognizes CheVs.

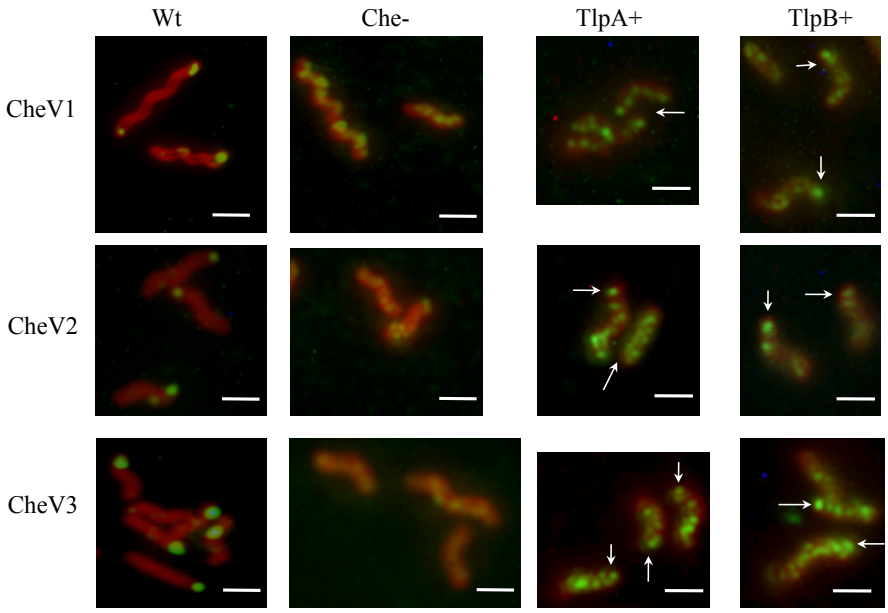

Figure 2d. The chemoreceptors, TlpA and TlpB, are required for the distinct polar localization. In isogenic mutants having a single chemoreceptor, the three CheV proteins are localized at the cell pole and diffused throughout the bacterial cell (polar and diffuse). The white arrow indicates the polar localization of each CheV protein. Naming is as follow: Wild type (Wt), strain with no chemoreceptors (Che-), strain with only TlpA (TlpA+), and strain with only TlpB (TlpB+).

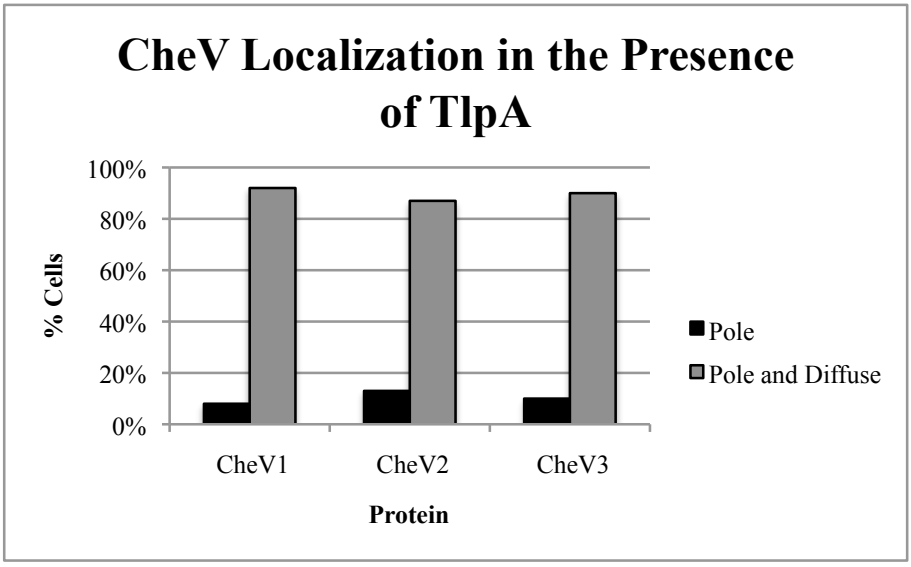

Figure 2e. Single-cell quantification of subcellular immunofluorescence localization of CheV1 CheV2 and CheV3. In a TlpA only strain, 92% of cells showed CheV1at the pole and diffused, 87% of cells showed CheV2 at the pole and diffused, and 90% of cells showed CheV 3 at the pole and diffused. The cell distribution data presented in the graph were obtained from cells represented in figure 2d treated with specific Alexa Fluor and visualized by fluorescence microscopy. The quantification was performed on at least 150 cells per experiment.

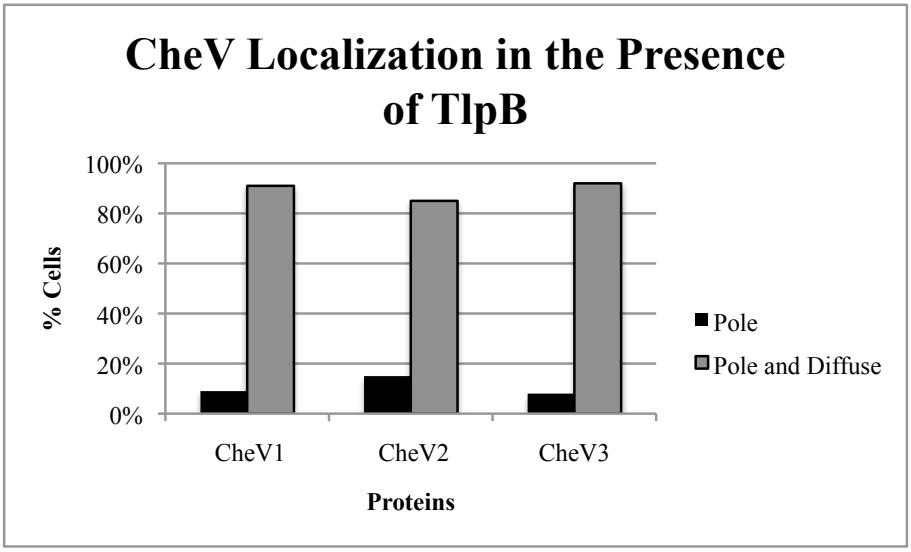

Figure 2f. Single-cell quantification of subcellular immunofluorescence localization of CheV1 CheV2 and CheV3. In a TlpB only strain, 92% of cells showed CheV1at the pole and diffused, 87% of cells showed CheV2 at the pole and diffused, and 90% of cells showed CheV 3 at the pole and diffused. The cell distribution data presented in the graph were obtained from cells represented in figure 2d treated with specific Alexa Fluor and visualized by fluorescence microscopy. The quantification was performed on at least 150 cells per experiment.

CheVs polar localization are chemoreceptor dependent

The findings presented above suggest that chemoreceptors play a key role in sequestering the three CheV proteins to the cell pole, but we did not yet know the role of each CheV in guiding one another. To determine the role of each CheV, immunofluorescence was used to visualize the localization of each CheV protein in isogenic strains lacking another *cheV*. We observed that in the absence of CheV1, 90% of CheV2 and 97% of Chev3 where diffused, suggesting CheV1 aids polar localization (Fig. 3a,b). In contrast, loss of CheV2 or CheV3 did not affect the localization of the remaining two CheVs (Fig. 3a,b). Thus it seems that neither CheV2 nor CheV3 function to localize other CheVs.

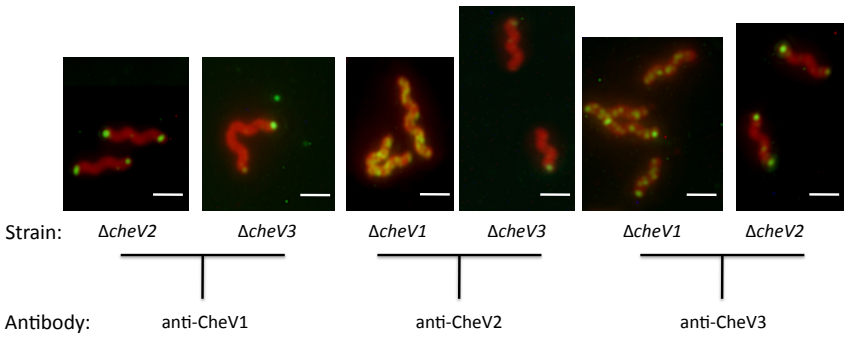

Figure 3a. CheV1 aids in polar CheV2 and CheV3 localization. Isogenic strains that lack CheV1, CheV2, and CheV3 (Δ *cheV1*, Δ *cheV2*, Δ *cheV3*) were probed with native antibody specific to CheV1, CheV2, and CheV3.

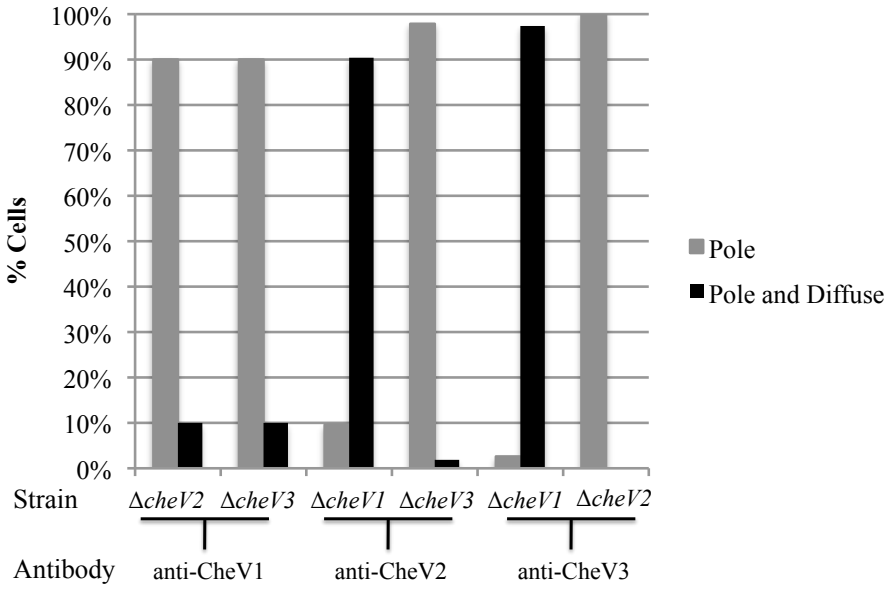

Figure 3b. CheV1 guides polar CheV2 and CheV3 localization. In the absence of CheV1, a majority of cells had 90% of CheV2 and 97% of CheV3 localized at poles and diffused. On the contrary, the *cheV*2 and *cheV*3 mutants did not affect the polar localization of the remaining CheV proteins and the phenotype resembled wild type localization as seen in figure 2b. The quantification was performed on at least 150 cells per experiment.

Since CheV1 affected the polar localization of both remaining CheV proteins, we asked whether CheV1 influenced the localization of the chemoreceptors. We reasoned that Δ*cheV1* mutation might disrupt the chemoreceptors and in turn lead to the mislocalization of CheV2 and CheV3. Immunofluorescence was thus applied to observe the role of CheV1 in localization of chemoreceptors. In wild type, 94% of bacterial cells had chemoreceptors localized at the cell poles (Fig. 4a,b). The Δ*cheV1* mutant, however, had a significant increase in staining throughout the entire cell (Fig. 4a). Quantification revealed that 92% of Δ*cheV1* cells had chemoreceptors localized both at the poles and diffused (Fig. 4b). We next asked whether the CheW coupling

protein would be necessary for polar chemoreceptor localization. Similar to the Δ*cheV1* mutant, a Δ*cheW* mutant had 96% of cells with chemoreceptors both at the pole and diffused (Fig. 4a,b). Thus both CheV1 and CheW stabilize the chemoreceptors at the poles. To check that Δ*cheV1*does not change the relative abundance of chemoreceptors, a western analysis was applied of wild type and the Δ*cheV1* strain (Fig. 4c). We observed no change in the amount of chemoreceptor present in the Δ*cheV1* strain, compared to wild type.

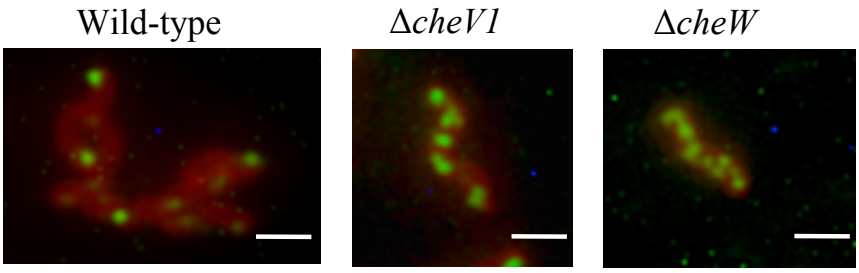

Figure 4a. The localization of chemoreceptors in wild-type and isogenic strains lacking CheV1, and CheW. Unlike the wild-type strain where chemoreceptors localized at the poles, the Δ*cheV*1 and Δ*cheW* mutant has a greater percentage of cells with chemoreceptors at the poles and diffused. For immunofluorescence the anti-GST-TlpA22 (antibody) was used for recognition of chemoreceptors (green/yellowish color).

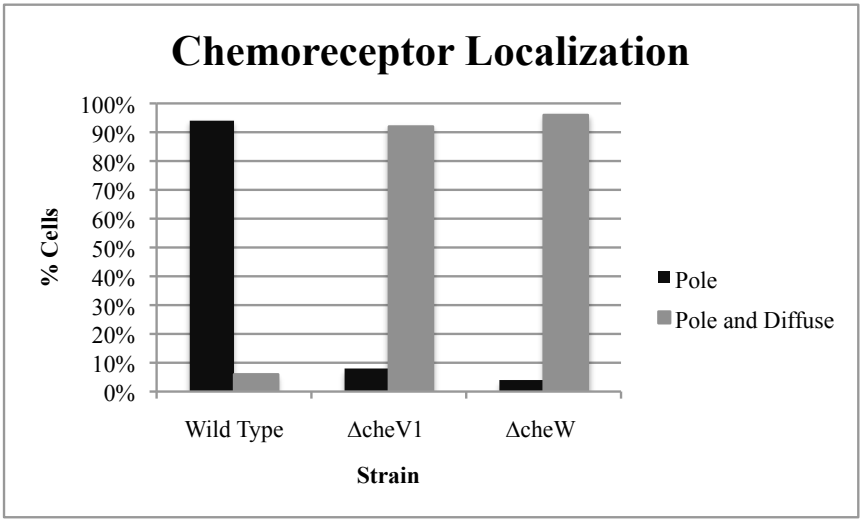

Figure 4b. The CheV1 and CheW chemotaxis proteins are required for the polar localization of the chemoreceptors. In wild type 94% of cells showed chemoreceptors to localize at the pole. Contrary to wild type the null *cheV1* strain had 8% of the cells with chemoreceptor localized at the poles. Similarly in the null *cheW* mutant 4% of the cells displayed chemoreceptors localized at the pole. The quantification was performed on at least 150 cells per experiment.

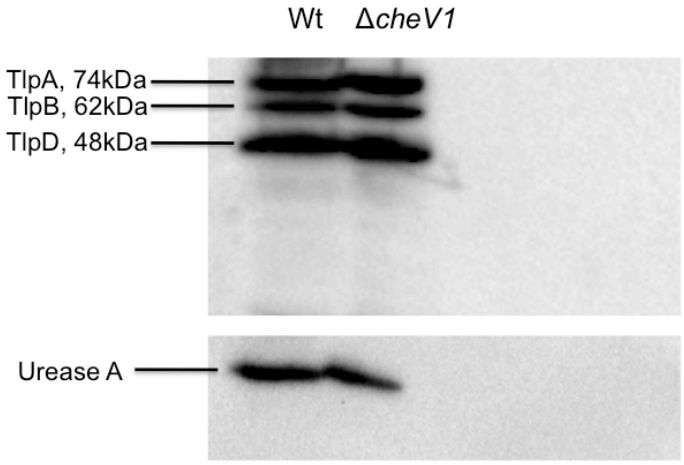

Figure 4c. In the absence of CheV1, no observable difference in chemoreceptors. Top panel depicts blot probed with anti-GST-TlpA22 anitbody (1:2000) and the detection of three chemoreceptors (TlpA, TlpB, and TlpD) in wild-type (Wt) and null c*heV1*. Cells normalized to and OD_{600} of 4.0 prior to acquiring cell lysates. For loading control, blot was striped prior to probing with anti-Urease A antibody (1:3000). The data is a representation of two experiments.

Discussion

The chemosensory machinery in bacteria has an important function in interpreting the surrounding environment. In *H. pylori*, we report here that the chemotaxis proteins CheV1, CheV2, and CheV3 do not have a chemoreceptor preference. All three CheV proteins were detected in membrane fractions of strains with only a single chemoreceptor present, TlpA or TlpB, by western analysis. Somewhat surprisingly, we also detected each CheV in membrane fractions of a strain without any chemoreceptors. This finding suggests that CheV proteins either associate non-specifically, or interact with a non-chemoreceptor complex protein. Similar observations have been made in *Bacillus subtilis*. Specifically, equal amounts of CheA was present in membrane fractions from strains missing the major chemoreceptors, CheW, CheR, CheB, and CheV (Garrity and Ordal 1997). It is known that the coupling protein, CheW, mediates the interaction between the histidine kinase, CheA, and chemoreceptors. Therefore, CheV proteins possibly interact with CheA that is retained in membrane fractions of a strain missing all the chemoreceptors. The western analysis of membrane fractions of single chemoreceptors showed that CheV proteins are detected regardless of TlpA or TlpB presence. We then decided to apply a different study to observe the spatial localization of the three CheVs (CheV1, CheV2, CheV3).

Immunofluorescence microscopy methodology was applied to show the spatial localization of the three CheV chemotaxis proteins. In wild type and a strain

missing all chemoreceptors, the CheV proteins were distinctively localized at the cell poles and dispersed throughout the cell, respectively. The latter suggests that CheV proteins interact with the other members of the chemoreceptor cluster, which are localized to the cellular pole (Lertsethtakarn thesis). We therefore examined strains that bore only a single chemoreceptor for all three CheV proteins localization. In the presence of a single chemoreceptor, TlpA or TlpB, the CheV proteins were polar and diffused. The apparent and distinct polar localization of CheVs seen in wild type was absent, however, there was more polar staining than in strains that completely lacked chemoreceptors. This finding indicated that CheV proteins do not prefer TlpA or TlpB. In fact the data imply that both populations of chemoreceptors, and perhaps TlpD are required for polar localization of the three CheVs. In wild-type bacteria, TlpA, TlpB and possibly TlpD appear to form a functional chemoreceptor array or clusters similar to other chemotactic bacteria (Bray, Levin et al. 1998; Wadhams and Armitage 2004; Briegel, Ortega et al. 2009). These polar chemoreceptors nucleate the formation of a chemotaxis supermolecular complex at the poles that involves chemoreceptors, CheA, CheW, CheY, and CheV proteins. On the contrary, the single chemoreceptor mutants might have a distorted chemoreceptor array that leads to improper ternary formation, or may just have fewer CheV docking sites, which explains the localization phenotype of the three CheV.

We showed the role of chemoreceptor arrays or chemoreceptor populations in localizing CheVs to the cell pole, however, the role of each CheV in mediating the localization of one another was unknown. Here we concluded by

immunofluorescence microscopy that CheV1 has a role in guiding CheV2 and CheV3 to the cell pole. To further elucidate the role of CheV1 we were curious to see the localization of the chemoreceptors in the absence of CheV1. We found that chemoreceptors were localized at the pole and diffused in the mutant. This phenotype differed from that of wild-type, where chemoreceptors are primarily found at the pole. Moreover, the result led us to conclude that CheV1 in wild-type has a role in localizing and possibly stabilizing the chemoreceptor array to the pole. Furthermore, our findings suggest that the polar localization of CheV2 and CheV3 are lost in the absence of CheV1 because of a non-wild-type chemoreceptor array. One can also imply that CheV2 or CheV3 do not affect chemoreceptor localization, because they do not have a role in the localization of one another. Next we looked for the localization of chemoreceptors in the absence of the coupling protein, CheW. By using the same approach we saw the same localization pattern as in the CheV1 mutant, which indicates that both CheW and CheV1 are needed for the polar localization of the chemoreceptors. The mechanism on how CheV1 and CheW guide chemoreceptors to the pole remains to be answered.

This study reveals a novel role of CheV1 in the localization of the chemoreceptors in *H. pylori*. We showed that the three CheV proteins do not have a chemoreceptor preference. Furthermore, our observations showed that CheV1 guide the localization of the other two CheVs by affecting the polar localization of the chemoreceptors (see model Figure 5). It is CheV1 and CheW that help to localize the chemoreceptors to the cell pole. Work from *B. subtilis* examined how loss of *cheW*

alone or in combination with loss of *cheV* affected receptor clustering (Lamanna, Ordal et al. 2005). These authors report that 85% of wild type cells had clusters of the McpB chemoreceptor. When *cheW* was deleted, the percentage with clusters decreased to ~75%. With both *cheW* and *cheV* eliminated, the percentage of cells with clusters dropped to ~50%. Our studies here showed a major decrease in chemoreceptor polar localization in the absence of CheV1 and CheW, too. Which leads us to believe that both chemoreceptor populations at the cell pole contribute to the formation of the sensory/ternary complex. The correct spatial localization of all chemotaxis signaling proteins is important for the formation of the large sensory complex, which helps to monitor and respond appropriately to the environment (Maddock and Shapiro 1993; Sourjik 2004; Greenfield, McEvoy et al. 2009; Zhang, Liu et al. 2012). Our results suggest a similar localization and formation pattern takes place in the gastric pathogen *H. pylori* as well. In fact a previous study showed that a mutant of CheV1 had a severe chemotactic and colonization defect (Lowenthal, Simon et al. 2009). Our studies presented here add another explanation to the observations seen by Lowenthal's study. Where the mutant CheV1 and CheW fail to lead the chemoreceptors to the cell pole, which then results in a distorted chemoreceptor array that is unable to form an appropriate chemosensory complex. Therefore the pathogen is unable to properly sense the environment, which then leads to chemotaxis and colonization defects. In a complete sensory complex, where the CheVs and other chemotaxis signal transduction proteins are localized at the pole, *H.*

pylori can better assess the surrounding environment and respond by moving toward conditions that promote its survival.

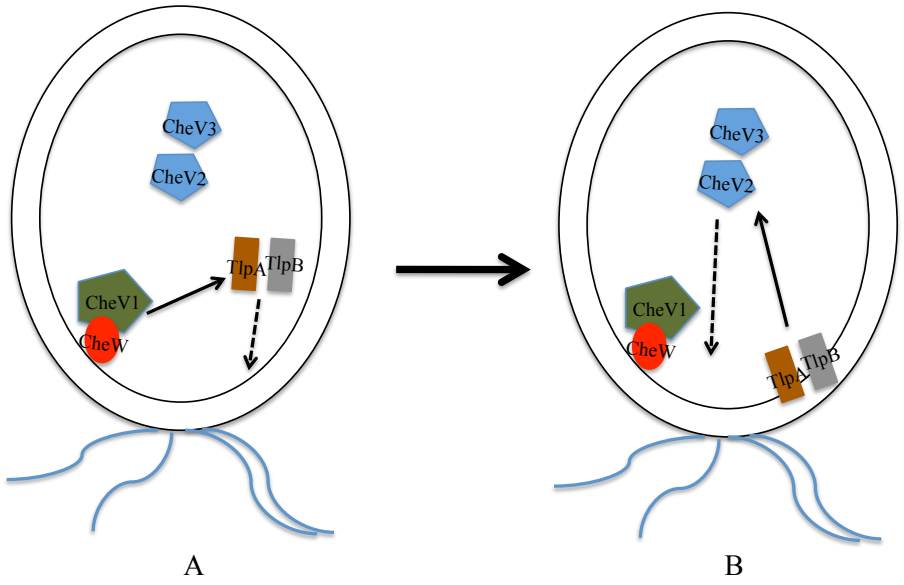

Figure 5. Proposed working model for CheV1 leading the polar localization of chemotaxis signaling proteins in *H. pylori*. In figure A, CheV1 leads (black arrow) the chemoreceptors to the cell pole (dashed black arrow). Once at the pole the chemoreceptors guides (black arrow) the remaining CheVs to the cell pole (black dashed arrow); figure B. The formation of the large sensory complex helps the gastric pathogen to monitor and respond appropriately to the host environment. The *H. pylori* chemotaxis signaling proteins, CheZ and CheAY, are not shown in the model.

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