Attractant Binding Induces Distinct Structural Changes to the Polar and Lateral Signaling Clusters in *Bacillus subtilis* Chemotaxis

Kang Wu, Hanna E. Walukiewicz, George D. Glekas, George W. Ordal, and Christopher V. Rao

From the Departments of Chemical and Biomolecular Engineering and Biochemistry, University of Illinois at Urbana-Champaign, Urbana, Illinois 61801

Bacteria employ a modified two-component system for chemotaxis, where the receptors form ternary complexes with CheA histidine kinases and CheW adaptor proteins. These complexes are arranged in semi-ordered arrays clustered predominantly at the cell poles. The prevailing models assume that these arrays are static and reorganize only locally in response to attractant binding. Recent studies have shown, however, that these structures may in fact be more fluid. We investigated the localization of the chemotaxis signaling arrays in *Bacillus subtilis* using immunofluorescence and live cell fluorescence microscopy. We found that the receptors were localized in clusters at the poles in most cells. However, when the cells were exposed to attractant, the number exhibiting polar clusters was reduced roughly 2-fold, whereas the number exhibiting lateral clusters distinct from the poles increased significantly. These changes in receptor clustering were reversible as polar localization was reestablished in adapted cells. We also investigated the dynamic localization of CheV, a hybrid protein consisting of an N-terminal CheW-like adaptor domain and a C-terminal response regulator domain that is known to be phosphorylated by CheA, using immunofluorescence. Interestingly, we found that CheV was localized predominantly at lateral clusters in unstimulated cells. However, upon exposure to attractant, CheV was found to be predominantly localized to the cell poles. Moreover, changes in CheV localization are phosphorylation-dependent. Collectively, these results suggest that the chemotaxis signaling arrays in *B. subtilis* are dynamic structures and that feedback loops involving phosphorylation may regulate the positioning of individual proteins.

Many motile bacteria employ for chemotaxis a modified two-component system to sense and respond to chemicals, where the receptors form ternary complexes with the CheA histidine kinase and the CheW adaptor protein (1, 2). The clustering of these ternary complexes into semi-ordered hexagonal lattices has been documented in multiple species (3) and is presumably conserved in all chemotactic bacteria where the three proteins are found. These arrays are thought to amplify the response to attractant binding (4, 5). A number of models have specifically proposed that cooperative interactions between the receptors within these arrays enable bacteria to sense small differences in the number of attractant-bound receptors over a wide range of concentrations (see Ref. 6).

Multiple studies have investigated the structure and molecular determinants of these clusters (e.g. Refs. 7 and 8) along with their role in signal transduction. In *Escherichia coli*, the receptors form mixed trimers of receptor homodimers. These trimers are believed to form the basic building blocks for the larger clusters, which range in size from tens to thousands of receptors (9). These clusters are found predominantly at the cell poles, although they are also found along the lateral length of the cell. Attractant binding, which inhibits kinase activity in *E. coli* (10), increases the distance between the periplasmic sensory domains of the receptors dimers within the trimer; the distance between the intracellular signaling domains, however, remains unchanged and may even slightly decrease (11). Attractant binding also affects the clustering of the trimers, although the extent and magnitude of these changes vary depending on the study.

In one study (12), the binding of nearly saturating concentrations of attractant was found to disrupt the receptor clusters located at the cell poles in both *E. coli* and *Bacillus subtilis*. Prior to the addition of attractant, the receptors were found clustered primarily at the cell poles. Upon exposing the bacteria to saturating concentrations of attractant, the number of cells where the receptors were clustered at poles significantly decreased. However, once the cells adapted to the attractant, polar clustering was restored in the majority of the cells. These results support a model where attractant binding decreases the packing of the receptors within the semi-ordered lattices at the cell poles, leading to more fluid associations between the receptors and a reduction in the degree of clustering. Such a model would predict in *E. coli* that a reduction in the degree of clustering decreases kinase activity and *vice versa*. Consistent with this model, the activity of the CheA kinase in *E. coli* was found to increase as the density of receptors increases (13, 14). In further support of this model for clustering and kinase regulation, multivalent ligands that destabilized the clusters in *E. coli* behaved as attractants.
whereas ones that stabilized the clusters behaved as repellents (15, 16).

Although the above results support a model where attractant binding reduces receptor clustering, others have shown in E. coli that attractant binding does not affect clusters and causes only local changes to the arrangement of the receptors within the clusters (17, 18). This alternate model is further supported by studies where receptor modifications that increase or decrease kinase activity were found to lead to only minor changes in receptor clustering, far less than what would be expected based on the change in kinase activity or as suggested by the model described in the preceding paragraph (17, 19–21).

In addition to clustering, signaling may also be governed by interactions between the receptors and the kinase. Support for such a mechanim comes from CheV, a hybrid protein with an N-terminal CheW-like adaptor domain and a C-terminal response regulator domain known to be phosphorylated by CheA (22–25). This protein is functionally similar to CheW and is believed to function in a separate, methylation-independent adaptation system (23, 26). First discovered and characterized in B. subtilis (23, 27), CheV has subsequently been found in numerous other bacteria including Salmonella enterica but not E. coli (28–31). Interestingly, in the gastric pathogen Helicobacter pylori, the single adaptation system employs three CheV paralogues but not reversible receptor methylation (24, 32). Despite its prevalence, little is known about how this protein functions in chemotaxis beyond the methylation (24, 32). Despite its prevalence, little is known about how this protein functions in chemotaxis beyond the requirement for phosphorylation.

In the present study, we explored the clustering of the receptors and CheV in B. subtilis during the excitation and adaptation to attractant. Our results demonstrate that prior to excitation with attractant, the receptors form clusters mostly at the cell poles. When the cells were initially excited with attractant, we observed a reduction in the number of receptor clusters at the pole and an increase in the number on the sides of the cell. When the cells adapted to the attractant, we again found that the receptors cluster mostly at the poles of the cell. Interestingly, we found that CheV is predominantly localized at lateral clusters on the sides of the cell prior to stimulation with attractant. Upon excitation with attractant, however, we found that CheV was predominantly localized at the poles in the majority of cells. This transition is reversible as CheV was found to be localized predominantly in lateral clusters once the cells adapt. In addition, the redistribution of CheV is phosphorylation-dependent; deleting the response regulator domain or mutating the phosphorylation site causes CheV to localize primarily at the cell poles, irrespective of attractant. Collectively, these results provide new insights regarding receptor clustering and the CheV adaptation system in B. subtilis chemotaxis and possibly other species of bacteria as well.

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains and Growth Conditions**—Basic cloning steps were performed in E. coli strain DH5α. The E. coli K12 dam- strain GM2929 (33) was used for plasmid propagation when the enzyme cutting site ClaI was used. All strains and plasmids used in this study are listed in Table 1. E. coli was grown in Luria-Bertani (LB) media at 37 °C. B. subtilis was grown in minimal media (50 mM K2PO4, 1.2 mM MgCl2, 1 mM (NH4)2SO4, 140 μM CaCl2, 10 μM MnCl2, 20 μM sorbitol, 50 μg/ml histidine, 50 μg/ml methionine, and 50 μg/ml tryptophan). When necessary, the antibiotics ampicillin and spectinomycin were used at concentrations of 100 μg/ml.

**Plasmid and Strain Construction**—The plasmid, pKW244, carrying the mcpB-gfpmut3 fusion was built in three steps. First, the gfpmut3 gene was amplified by PCR using pGFPmut3 (34) as the template and inserted into the ClaI and SpeI restriction sites of plasmid pMUTIN-GFP+ (35), replacing gfp with gfpmut3 and yielding the plasmid pKW235. Next, the erythromycin resistance marker on pKW235 was replaced with a spectinomycin marker. The gene encoding for a spectinomycin resistance was amplified using the plasmid pDG1664 as the template with primers containing overhanging BsfI restriction sites. The plasmid pKW235 excluding the erythromycin resistance gene was amplified with primers containing overhanging BsaI restriction sites. These two fragments were ligated together after enzyme digestion, resulting in the plasmid pKW238. This plasmid can be used to construct a fusion of GFPmut3 to the C terminus of any protein in B. subtilis. To
fuse GFPmut3 to McpB, the last 300 bp of the mcpB gene was amplified and cloned into the KpnI and Clal restriction sites of pKW238, resulting in the plasmid pKW244. The plasmid pKW244 carrying the mcpB-gfpmut3 fusion was integrated into the mcpB locus of OI1085, yielding the strain OI4454. The strain OI3635 (36) was then transformed with OI4454 genomic DNA and selected with spectinomycin, yielding the strain OI4455. The McpB-GFP fusion protein was shown to be functional on motility plates.

Immunofluorescence Staining—Cells were grown overnight in minimal media at 37 °C and then diluted 1:10 in 1 ml of minimal media and subcultured to $A_{600} = 0.5–0.6$. Bacteria were fixed for 15 min at room temperature and then 30 min on ice in 2.5% (v/v) paraformaldehyde (Ted Pella, Redding, CA). Fixed bacterial cells were then plated onto poly-L-lysine-coated glass coverslips and incubated for 10 min. The coverslips were then washed three times (for 5 min each) in phosphate-buffered saline (PBS) containing 2% (w/v) bovine serum albumin (BSA). The cells were treated with lysozyme (0.1 μg/ml) for 20 min. The coverslips were washed again three times (for 10 min each) with PBS containing 2% BSA (w/v) and incubated overnight at 4 °C in primary antibody diluted into PBS containing 2% (w/v) BSA. The primary antibody anti-McpB was used at a dilution of 1:500, anti-CheA was used at a dilution of 1:2000, anti-CheV was used at a dilution of 1:1000, and anti-CheW was used at a dilution of 1:5000. These custom rabbit polyclonal antibodies were raised against purified chemotaxis proteins (Cocalico Biologicals). All antibodies were pretreated six times by acetone powder preabsorption using their corresponding knock-out strains: OI3545 (∆10mcp) for anti-McpB, OI1840 (∆cheA) for anti-CheA, OI3059 (∆cheV) for anti-CheV, and OI2737 (∆cheW) for anti-CheW to reduce background signals caused by nonspecific binding. After overnight incubation with the primary antibodies, the coverslips were then washed three times (for 10 min each) with PBS containing 2% (w/v) BSA and then incubated for 5 h at 4 °C in with a goat anti-rabbit antibody labeled with FITC (Molecular Probes) at a dilution of 1:500 in PBS containing 2% (w/v) BSA. Finally, the coverslips were washed three times (for 5 min each) in PBS containing 2% (w/v) BSA and mounted onto slides using ProLong with DAPI (Molecular Probes). ProLong was allowed to dry completely, eliminating the need to seal the coverslips to the slides.

Fluorescence Microscopy—Fixed cells were imaged using a Zeiss Axiovert 200M inverted microscope equipped with a 100× objective and a Zeiss Apotome structured illumination system. Images were acquired using a Roper Scientific Cascade 512B EM CCD camera. In each field of view, 0.25-μm optical sections were collected through the z axis. Each image was then deconvoluted using AutoQuant X software (MediaCybernetics).

For live cell imaging, cells were grown under the same conditions to $A_{600} = 0.5–0.6$. A 1–2-μl aliquot of culture was placed on a coverslip under a piece of agar pad (1 mm thick, 1.5% (w/v) agar in PBS). Imaging was performed using an inverted epifluorescence microscope (Eclipse TE2000-E; Nikon) with a 100× objective (Plan Fluor, numerical aperture 1.40). Images were acquired using a Photometrics Cascade 512 cooled CCD camera. Acquisition was performed using MetaMorph software (Molecular Devices).

Image Analysis—Analysis was performed using the AutoQuant X software package (MediaCybernetics). Each cell was treated individually for analysis purposes. First, cells were identified by the presence of both a DAPI and an FITC signal, where the latter indicated that the target protein was present (Fig. 1). In the event that either the DAPI or the FITC signal was missing, the cell was eliminated from further analysis. Next, once a candidate cell was identified, the DAPI signal was analyzed along the longitudinal axis of the cell, denoted by the line X (supplemental Fig. S1). If distinct and clearly demarked DAPI peaks were observed along the X line, then each peak was treated as an individual cell. If multiple DAPI peaks without clear demarcation boundaries were observed, then the candidate cell was eliminated from further analysis. Next, the cell was divided into six equal pieces along the longitudinal axis. These lines denote the traverse axis of the cell and are labeled Y1 through Y5, respectively (supplemental Fig. S2). When the length of the cell could not be precisely determined, the lines Y1 and Y5 were chosen such that the peak DAPI signal along Y1 and Y5 lines was ~25% of the peak DAPI signal along the X line. Cells were classified as having polar, lateral, diffuse, or both polar and lateral localization based on the fluorescence intensity observed along the X and Y1–Y5 lines (supplemental Fig. S2). First, the FITC signal was examined along the X and Y1–Y5 lines. If the FITC signal was present along all quantification lines, then the cell pattern was classified as diffuse. If the FITC fluorescence showed two peaks along the X line, as

Given the document and the expected output, here is the formatted text:

**Cellular Localization in B. subtilis Chemotaxis**

**A**

![Image](Image 337x505 to 541x734)

**B**

| Polar | Lateral | Diffuse | Both |
|-------|---------|---------|------|

**FIGURE 1.** Chemotaxis protein localization visualized by immunofluorescence microscopy. **A,** a field of view showing the localization of McpB by immunostaining. The blue signal is from DAPI, indicating the location of the DNA, and the green signal is from FITC, indicating the location of the target protein. The scale bar shown is 3 μm. **B,** examples showing different CheV localization patterns. The bacteria were incubated with preabsorbed anti-CheV antiserum (diluted 1:1000). The sample was then treated with secondary FITC-conjugated goat anti-rabbit antibody (green) and DAPI (blue). Cell outlines denoted by dashed lines are not exact and shown for better visualization. The scale bar shown is 1 μm.
well as one or more peaks along the Y1 line and/or one or more peaks along the Y5 line but no peaks along the Y2–Y4 lines, then the cell pattern was classified as polar. For the purpose of this study, no distinction was made between cells exhibiting a “polar cap” and cells exhibiting two or more distinct spots localized to the poles (see Ref. 37). Likewise, no distinction was made between cells that exhibited FITC signal at just one pole versus those that had FITC signals at both poles. In other words, we did not distinguish between cells having a single polar cluster at one end versus cells having them at both ends (see Ref. 38). If the FITC signal showed one or more peaks along the X line and no peaks along the Y1 or Y5 lines along with a peak in any or all of Y2–Y4 lines, the localization pattern was classified as lateral. If the FITC signal showed two or more peaks along the X line and showed one or more peaks along the Y1 and/or Y5 lines, along with one or more peaks along the Y2–Y4 lines, then the localization pattern was classified as both polar and lateral. For each experiment, at least 100 cells were analyzed at random. All experiments were performed on three separate days with the averages and S.D. reported.

RESULTS

Attractant Binding Reduces the Number of Polar Receptor Clusters and Increases the Formation of Lateral Clusters—We first imaged receptor localization in B. subtilis using immunofluorescence. Our approach was similar to the one employed by Lamanna et al. (12), although we refined their analysis to take into account recent observations in E. coli regarding the positioning of the receptor clusters. Although the majority of receptors are localized at the poles in E. coli, many are also found in distinct clusters laterally along the length of the cell (7–9, 37, 39–41). Consistent with previous results (12, 42), we found that McpB, the sole chemotaxis receptor for the attractant asparagine (43), was localized at the cell poles in the majority of B. subtilis cells (Fig. 2). However, when the cells were exposed to nearly saturating concentrations of asparagine (1 mM), we observed a significant reduction in the number of cells where McpB was clustered at the poles and an increase in the number where it was clustered on the sides of the cell (Fig. 2). Once the cells were allowed to adapt, polar localization was restored. These results suggest that asparagine binding not only destabilizes the polar clustering of McpB, as observed previously, but also induces the formation of lateral clusters by some unknown mechanism.

Differences between the present and previous results are likely due to how the cells were prepared and the fluorescence images collected and processed. In particular, we included an additional blocking step prior to incubating the permeabilized cells with antibody. We also preabsorbed the antibody several times against the corresponding knock-out strain to reduce nonspecific binding. These steps significantly reduced background fluorescence. In addition, we acquired our images as Z-stacks and were thus able to reduce the background signal and noise by three-dimensional deconvolution. Collectively, these modifications afforded higher resolution imaging of the cells, enabling us to observe patterns of receptor localization not otherwise possible (Fig. 3). Specifically, the diffuse staining previously observed is likely due to the formation of lateral clusters. As a consequence, we were able to extend and refine the observations concerning McpB localization made by Lamanna et al. (12).

Live Cell Fluorescence Microscopy Also Demonstrates That Attractant Binding Reduces the Number of Polar Receptor Clusters and Increases the Formation of Lateral Clusters—Studies where attractant binding was found to reduce polar receptor clustering employed immunofluorescence (12, 15). Those studies that failed to observe any changes in receptor clustering employed live cell microscopy and fluorescent protein fusions (17, 18, 21). To test whether our results were an artifact of immunofluorescence, we also performed analogous experiments in cells where the green fluorescent protein was fused to McpB (McpB-GFP). As B. subtilis adapts to asparagine in less than 1 min, we performed the live cell fluorescence experiments in a strain that does not adapt to the addition of attractant. As a consequence, any change in receptor localization due to the addition of attractant would persist in these cells. The reason that this is important is that the results are not affected by the time it takes to image the cells.
Consistent with our results tracking receptor localization in wild-type cells using immunofluorescence, we found using live cell fluorescence that the addition of asparagine reduced the number of cells where McpB fused to the green fluorescent protein (GFP) was clustered at the cell poles and increased the number where it was clustered in lateral clusters at the sides of the cell (Fig. 4). Representative images of these results are given in supplemental Fig. S3. Similar results were also obtained using immunofluorescence in otherwise identical cells expressing the wild-type, untagged McpB (Fig. 4B). These results indicate that the observed changes in receptor clustering are not an artifact of immunofluorescence.

Attractant Binding Reduces the Extent of CheV Localization at Lateral Clusters and Increases It at Polar Clusters—CheW is a scaffold protein, found in all chemotactic bacteria, that facilitates the coupling between CheA and the chemotaxis receptors (44, 45). CheV, a hybrid protein consisting of an N-terminal CheW-like domain and a C-terminal response regulator domain, is functionally redundant to CheW in B. subtilis; loss of either protein does not prevent adaptation but does lead to a minor reduction in chemotaxis efficiency (23, 27). Loss of both proteins, however, completely abrogates chemotaxis. Previously, we hypothesized that CheV functions in a methylation-independent adaptation system where the phosphorylation of its response regulation domain inhibits the coupling between CheA and the receptors (26). Such a model could explain how CheV functions in a distinct adaptation system. However, this model was formulated under the assumption that receptor clusters are static entities as opposed to in fact being dynamic ones and thus is incomplete.

Our previous results regarding the localization of the receptors led us to hypothesize that CheV may somehow be involved in this process. To image CheV localization, we again used immunofluorescence, employing the same procedure used to track the localization of McpB. We also attempted to track the localization of CheV using live cell fluorescence. However, CheV fusions to GFP were not functional, as determined using motility swarm plates. As our immunofluorescence results for McpB are the same as those obtained using live cell fluorescence, there was no reason to believe that they would be different for CheV.

To our surprise, we found that CheV was not localized at the poles in most cells but rather at lateral clusters (Fig. 5). Remarkably, when the cells were stimulated with asparagine, we found that CheV was clustered at the poles in most cells. Once the cells adapted, CheV was again mostly localized in lateral clusters. These results indicate that the changes in
CheV localization and clustering in response to the addition of attractant are opposite to those observed for McpB.

To determine whether CheV has an effect on the receptors, we tracked the localization of McpB in a cheV-null mutant (Fig. 6). Comparison with our wild-type results showed that there was a mild decrease in the number of cells exhibiting polar clusters. These polar clusters, however, were more stable in the cheV-null mutant as fewer cells exhibited lateral clusters or diffuse staining upon the addition of asparagine.

We also performed similar localization studies with CheW (supplemental Fig. S4). Unlike CheV, we found that CheW was predominantly localized at the cell poles. Moreover, the spatial distribution of CheW did not change upon exposure to asparagine. If anything, we saw an increase in the degree of polar localization. Collectively, these results suggest that CheV is not a static protein, as is the case with CheW, but rather a dynamic one that spatially redistributes during excitation and adaptation to asparagine.

**Changes in CheV Localization Are Phosphorylation-dependent**—During the initial characterization of CheV, phosphorylation of this protein was found to be necessary for adaptation. Mutants of B. subtilis where CheV cannot be phosphorylated, either because the phosphorylation site was mutated to an alanine residue or because the whole response regulator domain was deleted, do not adapt following the addition of attractant (23). We, therefore, hypothesized that the phosphorylation of the CheV response regulator domain may control its movement. Consistent with our hypothesis, we found that mutating the phosphorylation site to an alanine (Fig. 7) or deleting the response regulator domain in its entirety (supplemental Fig. S5) inhibits the redistribution of this protein upon stimulation by asparagine. In both mutants, we found that CheV was predominantly localized at the cell poles, irrespective of whether asparagine was added. Although some minor changes were observed in the case of the point mutant, deletion of the response regulator domain made CheV static. These results suggest that phosphorylated CheV has a greater tendency to associate with lateral clusters rather than polar ones; in contrast, unphosphorylated CheV tends to associate with the polar clusters rather than the lateral ones.

**CheW Inhibits the Transient Polar Localization of CheV**—As CheW and CheV are substantively redundant (23), we next tested how CheW affects CheV localization (Fig. 8A). Prior to stimulation with asparagine, we did not observe any significant difference in CheV localization patterns in a cheW-null mutant relative to the wild type. Upon stimulation with asparagine, we again observed that CheV was predominantly localized at the poles. However, we found that CheV was polarly localized in a slightly higher percentage of cells as compared with the wild type (52% versus 45%). These results suggest that CheW may hinder the movement of CheV to the poles by competitively inhibiting the binding of CheV to the receptors and CheA. When we performed reciprocal experiments, however, we found that CheV had no effect on the CheW localization (Fig. 8B); CheW was predominantly localized at the cell poles and was unaffected by asparagine in a cheV-null mutant.

**CheW but Not CheV or CheA Affects the Polar Localization of Receptor Clusters**—CheW is known to affect receptor clustering in E. coli and B. subtilis (7, 8, 12). Consistent with these previous observations, we found that McpB localized at polar
clusters in fewer cells when CheW was no longer present as compared with the wild type (Fig. 9). In addition, inspection of the McpB staining patterns revealed that the polar clusters were more diffuse in a cheW-null mutant and did not exhibit the punctate spots seen in wild-type cells (similar punctate spots are also observed in wild-type E. coli (8)). Conversely, loss of CheV or CheA was not found to affect polar localization (Fig. 9). As CheV is predominantly localized at the lateral clusters and not the polar ones, these results are entirely expected; deleting the protein would not be expected to affect the polar clusters. In the case of CheA, we found that the loss of this protein did not affect polar localization, although inspection of these clusters revealed that they were more diffuse. Similar results are also observed in E. coli (8, 46).

**Attractant Binding Does Not Affect CheA Localization**—As CheA was found to be primarily localized to the cell poles, we last tested how its localization may be affected by attractant binding. In particular, we sought to determine whether the binding of asparagine affects the localization of CheA. Our results indicate that it does not. We found that CheA was primarily localized to the cell poles in the absence of asparagine (supplemental Fig. S6). Upon the addition of asparagine, we observed a small reduction in the number of cells where CheA was clustered at the poles and an equivalently small increase in the number where CheA was clustered at the sides of the cell. Once the cells adapt, prestimulus localization patterns were restored. Similar results were also observed when CheA localization was tracked in cells expressing only McpB (data not shown). Collectively, these results indicate that CheA is mostly a static protein and that its localization is only weakly affected by the addition of saturating asparagine.

**DISCUSSION**

Multiple studies have observed lateral signaling clusters in E. coli (7–9, 37, 39, 40). Whether these lateral clusters play a role distinct from the polar ones in E. coli is still unknown, although the evidence to date suggests that they do not. In the present study, we made the unexpected discovery that the structure and composition of the polar and lateral clusters in B. subtilis change during chemotaxis toward asparagine.
These results suggest that polar and lateral clusters may play distinct roles in *B. subtilis* chemotaxis.

Specifically, we found that adding nearly saturating concentrations of asparagine reduces the fraction of cells where McpB at the poles forms visible clusters. Interestingly, adding asparagine increases the fraction of cells where McpB on the sides forms visible clusters. This process is reversed once the cells adapt, with the clusters becoming visible again at the poles and disappearing on the sides. These results demonstrate that the receptors respond differently to attractant binding depending on whether they are located at the poles or on the sides of the cell.

In addition, we found that CheV, a chemotaxis protein found in *B. subtilis* but not *E. coli* (23), is predominantly clustered on the sides of most cells prior to stimulation with asparagine. When the cells are stimulated with asparagine, CheV is found primarily clustered at the cell poles in a pattern opposite to what is observed with the receptors. As with the receptors, this process is reversible with CheV found again predominantly clustered on the sides in adapted cells. Most intriguingly, we found that the localization of CheV is phosphorylation-dependent, where mutation of the CheV phosphorylation site or deletion of its response regulator domain fixes the protein at the cell poles (23).

These results were only possible because of how we imaged the cells and analyzed the data. In particular, we would not have observed the reported patterns of receptor localization otherwise. Specifically, many changes are subtle and become apparent only when analyzing large numbers of cells. Clearly, a number of questions remain unanswered. Nonetheless, these findings are significant as they demonstrate that the polar and lateral signaling clusters undergo distinct and often reciprocal structural and compositional changes during chemotaxis in *B. subtilis*. In addition, the process is phosphorylation-dependent.

Regarding CheV, this protein is involved in a methylation-independent adaptation system in *B. subtilis* and other bacteria as well (25, 45). Previously, the phosphorylation of CheV was thought to disrupt the coupling between the receptors and CheA (26). Such a mechanism would explain how CheV functions within adaptation, namely by functioning within a phosphorylation-dependent negative feedback loop. However, this model was formulated under the assumption that CheV is a static protein.

The simplest interpretation of our data would suggest that CheV is not a static protein but instead reversibly traffics from the sides to the poles of the cell. Most likely, phosphorylation controls this process. In both unstimulated and adapted cells, CheV is found predominantly in lateral clusters. Likewise, mutants of CheV that cannot be phosphorylated are fixed at the cell poles. This would suggest that phosphorylated CheV preferentially binds to the receptors at the sides, whereas unphosphorylated CheV preferentially binds at the poles. Additional factors likely control the localization of CheV. How the reversible trafficking of CheV forms part of a methylation-independent adaptation system, however, is unknown.

Regarding the receptors, we note that multiple studies have investigated how attractant binding affects clustering (12, 17, 18, 21). As discussed previously, our observed changes in receptor clustering are entirely consistent with those made by Lamanna *et al.* (12). However, in the case of *E. coli*, others failed to see any discernible changes in receptor clustering in response to the addition of attractant (17, 18, 21). Possibly, this discrepancy may be due to the fixation protocol used to study localization. To control for this, we made McpB-GFP fusion proteins and observed the same behavior when we imaged live cells expressing McpB fused to GFP, indicating that both our results and those of Lamanna *et al.* (12) are not artifacts of fixation.

One final outstanding question concerns the mechanism by which the different receptor clusters form and disappear. Although it is tempting to imagine that the receptors reversibly traffic from the poles to the sides of the cell, such a mechanism is unlikely. The diffusion coefficient for individual chemotaxis receptors is ~0.01 μm²/s (47). This suggests that a single receptor would take roughly 25 s to travel 1 μm, roughly half the length of the cell or the average distance between the polar and lateral clusters. As a comparison, CheV, a soluble protein, would take roughly a 10th of a second to travel the same distance. To put these times in perspective, 5 s is roughly how long it took us to stimulate the cells with asparagine and then fix them. Even if these estimates for the receptors are overly conservative, reversible trafficking becomes even less plausible when one factors in the time required for multiple receptors to dissociate from one cluster and then aggregate to form a different cluster. Although we cannot discount an active mechanism for transport, a simpler explanation would be that the receptors do not move at all. In fact, all direct evidence indicates that the receptor clusters are stable over time scales far exceeding those required for reversible trafficking (47, 48).

A more plausible model, consistent with other studies, is that attractant binding affects the degree of receptor packing within the polar clusters, causing the receptors to separate at the poles to a degree that distinct clusters are no longer observable by fluorescence microscopy, but not so much that they completely diffuse away. Conversely, attractant binding causes the lateral clusters to become more densely packed, opposite to what it is occurring at the poles. When the cells adapt to asparagine, the reverse process occurs, with the clusters at the poles tightening and those on the sides becoming more diffuse.

Support for this model in part comes from CheA, which is clustered at the poles of cell. CheA clustering requires the receptors (data not shown). However, asparagine does not alter CheA clustering, even in cells expressing only McpB. If the receptors did in fact move away from the polar receptor clusters upon asparagine binding, then we would expect an associated reduction in CheA clustering at the cell poles. However, we found that CheA clustering was unaffected by asparagine including in cells expressing just McpB (data not shown). Similar arguments can also be made with CheW. Of course, this raises a second question. Why do CheA and CheW preferentially cluster with the receptors at the poles.
and not those on the side of the cell? It also demonstrates that a key feature distinguishing the polar and lateral receptor clusters from one another is their affinity for CheA and CheW as well.

In conclusion, we found that the chemotaxis signaling clusters are not static structures in *B. subtilis* but instead dynamic ones whose structure and composition change in response to the binding of asparagine. Moreover, we found that the localization of CheV is regulated by protein phosphorylation. Although we still do not fully understand the physiological significance of these changes, they nonetheless demonstrate that bacterial chemotaxis is far from a solved problem with many new complexities still to be elucidated.

Acknowledgments—We thank Victor Sourjik for helpful discussions. We also thank Jon Ekman and the staff at the Image Technology group at the Beckman Institute for help with microscopy and image analysis.

REFERENCES

1. Hazelbauer, G. L., Falke, J. I., and Parkinson, J. S. (2008) *Trends Biochem. Sci.* 33, 9–19
2. Rao, C. V., and Ordal, G. W. (2009) *Contribution Microbiol.* 16, 33–64
3. Besschetnova, T. Y., Montefusco, D. J., Asinas, A. E., Shrout, A. L., An-...