Molecular Microbiology (2005) **57**(3), 774–785 doi:10.1111/j.1365-2958.2005.04728.x

Large increases in attractant concentration disrupt the polar localization of bacterial chemoreceptors

Allison C. Lamanna,[1] George W. Ordal[2] and Laura L. Kiessling[1,3]*

[1]*Department of Biochemistry, University of Wisconsin at Madison, Madison, WI 53706, USA.*

[2]*Department of Biochemistry, Colleges of Medicine and Liberal Arts and Sciences, University of Illinois at Urbana-Champaign, Urbana, IL 61801, USA.*

[3]*Department of Chemistry, University of Wisconsin at Madison, Madison, WI 53706, USA.*

Summary

In bacterial chemotaxis, the chemoreceptors [methyl-accepting chemotaxis proteins (MCPs)] transduce chemotactic signals through the two-component histidine kinase CheA. At low but not high attractant concentrations, chemotactic signals must be amplified. The MCPs are organized into a polar lattice, and this organization has been proposed to be critical for signal amplification. Although evidence in support of this model has emerged, an understanding of how signals are amplified and modulated is lacking. We probed the role of MCP localization under conditions wherein signal amplification must be inhibited. We tested whether a large increase in attractant concentration (a change that should alter receptor occupancy from *c.* 0% to > 95%) would elicit changes in the chemoreceptor localization. We treated *Escherichia coli* or *Bacillus subtilis* with a high level of attractant, exposed cells to the cross-linking agent paraformaldehyde and visualized chemoreceptor location with an anti-MCP antibody. A marked increase in the percentage of cells displaying a diffuse staining pattern was obtained. In contrast, no increase in diffuse MCP staining is observed when cells are treated with a repellent or a low concentration of attractant. For *B. subtilis* mutants that do not undergo chemotaxis, the addition of a high concentration of attractant has no effect on MCP localization. Our data suggest that interactions between chemoreceptors are decreased when signal amplification is unnecessary.

Accepted 24 April, 2005. *For correspondence. E-mail kiessling@chem.wisc.edu; Tel. (+1) 608 262 0541; Fax (+1) 608 265 0764.

Introduction

The chemotaxis system of bacteria has been used as a model for elucidating the molecular details of signal transduction in general and two-component signalling specifically (Bourret *et al*., 1991; Parkinson, 1993; West and Stock, 2001). Chemotactic signals are initiated by the binding of molecules in the environment to transmembrane chemoreceptors. A two-component signalling system transforms information on attractant and repellent concentrations into a behavioural response (Gegner *et al*., 1992). Changes in receptor occupancy influence the phosphorylation of the histidine kinase CheA, which is linked to the chemoreceptors via the scaffolding protein CheW (Schuster *et al*., 1993) and CheV in *Bacillus subtilis* (Karatan *et al*., 2001). The response regulator CheY is a substrate of CheA, and the concentration of phospho-CheY is influenced by external conditions (Wylie *et al*., 1988). Phospho-CheY can interact with the flagellar motor, thereby modulating flagellar rotation between the clockwise (CW) and counterclockwise (CCW) directions (Barak and Eisenbach, 1992). Bacteria 'run' in the presence of attractants, as the resulting bias towards CCW flagellar rotation propels them in the direction of the highest attractant concentration. A bias towards CW rotation results in a 'tumbling' movement, allowing the bacteria to move away from repellents and sample the surrounding area for attractants.

Bacteria return to their pre-stimulus state through adaptation. Adaptation, which is regulated by methylation of the chemoreceptors, allows cells to respond to changing levels of chemoeffectors. The methyltransferase CheR catalyses the methylation of glutamate residues in the cytoplasmic domain of the chemoreceptors (Springer and Koshland, 1977; Burgess-Cassler *et al*., 1982; Zimmer *et al*., 2000). CheB, another target of CheA phosphorylation, attenuates methylation through the hydrolysis of methyl esters generated by CheR (Stock and Koshland, 1978; Thoelke *et al*., 1989; Bunn and Ordal, 2004). Because of the role of methylation in adaptation, the chemoreceptors are termed methyl-accepting chemotaxis proteins (MCPs).

Critical components of the chemotaxis machinery are conserved from *Escherichia coli* to *B. subtilis*, including CheW (Hanlon *et al*., 1992a), CheA (Fuhrer and Ordal, 1991) and CheY (Bischoff and Ordal, 1991). Additionally, the sequences of the cytoplasmic domains of the MCPs

are similar (Moual and Koshland, 1996). Accordingly, antibodies raised to the cytoplasmic domain of one species cross-react with the MCPs of other species (Alam and Hazelbauer, 1991; Morgan *et al*., 1993). Chemoreceptors are composed largely of α-helical regions; they exist as stable homodimers (Milligan and Koshland, 1988), and their cytoplasmic ends associate to form a four-helix bundle (Kim *et al*., 1999). Moreover, the MCP cytoplasmic domains can oligomerize into trimers of dimers (Kim *et al*., 1999; Studdert and Parkinson, 2004), and these oligomers have been implicated in signalling (Ames *et al*., 2002; Levit *et al*., 2002; Bunn and Ordal, 2003). Higher-order complexes of signalling components may also occur, as chemoreceptors have been observed to concentrate at the cell poles in an evolutionarily diverse group of bacteria and an archaeon (Maddock and Shapiro, 1993; Harrison *et al*., 1999; Gestwicki *et al*., 2000; Kirby *et al*., 2000). Thus, it appears that the structural features of the MCPs and their ability to localize within the cell are conserved throughout bacteria.

Protein components of the chemotaxis systems of many prokaryotic species are similar, yet there are differences (Bischoff and Ordal, 1992; Armitage and Schmitt, 1997). Some bacteria, including *B. subtilis*, express CheV, a CheW homologue with an additional response regulator (CheY-like) domain (Fredrick and Helmann, 1994; Rosario *et al*., 1994; Pittman *et al*., 2001). In *E. coli*, methanol release (demethylation) occurs only in response to repellents (negative stimuli) (Stock and Koshland, 1978), whereas methanol is released from *B. subtilis* in response to all stimuli (Goldman *et al*., 1982; Hanlon *et al*., 1992b; Zimmer *et al*., 2000). In *E. coli*, attractant binding leads to a decrease in CheA phosphorylation, a decrease in CheY phosphorylation, and a subsequent running response. Conversely, in *B. subtilis*, attractants elicit an increase in CheA phosphorylation, an increase in CheY phosphorylation and a corresponding running response (Garrity and Ordal, 1997). Despite these differences, similarities are preserved in the structures of the signalling pathway components, the cellular organization of the chemoreceptors and the mechanism by which signals are transduced.

It has been hypothesized that MCP–MCP interactions are important in the signalling process that leads to chemotaxis (Bray *et al*., 1998; Kim *et al*., 2002; Mello and Tu, 2003). Bacteria have the ability to sense and respond to very small changes (~5%) in chemoattractant over concentration ranges that span five to six orders of magnitude (Mesibov *et al*., 1973; Segall *et al*., 1986; Jasuja *et al*., 1999; Kim *et al*., 2001). Amplification of the signal is needed to afford behavioural changes in response to small changes in attractant concentration (Jasuja *et al*., 1999; Stock, 1999; Sourjik and Berg, 2002). Conversely, no amplification is required if the changes in attractant concentration are large. Signal magnification has been proposed to occur through a lattice-type network of MCPs (Bray *et al*., 1998; Shi and Duke, 1998; Duke and Bray, 1999). Recent theoretical (Mello and Tu, 2003; Albert *et al*., 2004) and experimental (Lybarger and Maddock, 2000; Ames *et al*., 2002; Gestwicki and Kiessling, 2002; Lamanna *et al*., 2002; Sourjik and Berg, 2002; 2004; Zimmer *et al*., 2002; Homma *et al*., 2004) results provide support for this model in both *E. coli* and *B. subtilis*. We demonstrated that stabilizing interactions between sugar-sensing MCPs can alter the signal output of serine-sensing MCPs, in both *E. coli* (Gestwicki and Kiessling, 2002) and *B. subtilis* (Lamanna *et al*., 2002). Still, a molecular-level understanding of how signals are amplified at low yet not at high attractant concentrations is lacking.

In models for signal amplification via a lattice, proposals that the extent of inter-receptor communication depends on ligand concentration have been advanced (Bray *et al*., 1998; Duke and Bray, 1999; Bray, 2002; Albert *et al*., 2004). A change in the extent of chemoreceptor–chemoreceptor interactions might contribute to the ability of bacteria to respond to chemoeffectors over a large dynamic range. To test whether attractants elicit changes in MCP localization, we visualized changes in the polar localization of MCPs in cells treated with a high concentration of attractant. In either *E. coli* or *B. subtilis*, we observed a disruption of the MCP polar lattice when the change in attractant concentration is large. Moreover, we found that unresponsive mutants or those with strongly impaired chemotactic responses do not give rise to changes in MCP localization under these conditions.

Results

Effects of chemoattractants on the labelling patterns of chemoreceptors

Chemoreceptor cross-linking via disulphide formation or cysteine- or lysine-reactive agents has been used to study the structure and localization of MCPs (Falke and Koshland, 1987; Milligan and Koshland, 1988; Lee *et al*., 1995; Hughson and Hazelbauer, 1996; Ames *et al*., 2002; Bunn and Ordal, 2003; Homma *et al*., 2004; Studdert and Parkinson, 2004). We employed paraformaldehyde as a cross-linking agent. Given the small size of the reactive species (formaldehyde), covalent links will occur only between closely associated proteins (Leong, 1994). We compared MCP localization in wild-type, mutant, chemoeffector-stimulated and adapted bacteria using paraformaldehyde fixation. To visualize chemoreceptor localization, we treated fixed cells with an antibody that reacts with the cytoplasmic portion of the MCPs (Alam and Hazelbauer, 1991; Kirby *et al*., 2000) and a fluorescent secondary antibody. Bacteria give rise to either a staining pattern indicative of polar chemoreceptor clusters (Mad-

dock and Shapiro, 1993) or a staining pattern in which no polar clusters were observed (Fig. S1). We labelled this latter staining pattern 'diffuse'. We scored the pattern of fluorescence using two separate methods: visual inspection using approaches similar to those described previously (Ames *et al*., 2002), and line scan analysis of the deviation in fluorescence intensity across the longitudinal axis of the cell. Both methods afforded similar results.

Previous reports indicate that 85% of *E. coli* cells exhibit polar MCP localization (Ames *et al*., 2002). Using an anti-MCP antibody, we assessed MCP localization in unstimulated *E. coli* cells. Our data indicate that the chemoreceptors are localized at the poles for most of the population, but 16% of the bacteria exhibit a diffuse staining pattern (Fig. 1). Thus, our results are consistent with data in the literature (Ames *et al*., 2002). To test whether attractants elicit changes in MCP localization, we exposed *E. coli* to a concentration of attractant that should result in greater than 95% occupation of the relevant chemoreceptor (Mesibov and Adler, 1972; Adler *et al*., 1973; Mesibov *et al*., 1973; Biemann and Koshland, 1994). Specifically, we treated *E. coli* with 1 mM attractant (glucose, serine or aspartate), fixed the cells immediately with paraformaldehyde and visualized MCP localization. At the high level of receptor occupation expected at this concentration (> 95% of the target receptor), signal amplification is not required. With 1 mM attractant, 50–60% of the cells exhibit diffuse staining. Thus, the MCPs in at least half of these cells are not localized at the poles.

Bacteria adapt to a constant concentration of a stimulant over time. We hypothesized that the polar lattice of chemoreceptors would be restored in bacteria that had undergone adaptation after exposure to a chemoattractant. To test this hypothesis, cells were exposed to high concentrations of chemoattractant (1 mM) over a time period sufficient to allow for adaptation before subsequent treatment with attractant (1 mM) and paraformaldehyde to induce cross-linking. The percentage of cells exhibiting diffuse staining under these conditions was similar to that of untreated cells (20–25%) (Fig. 1).

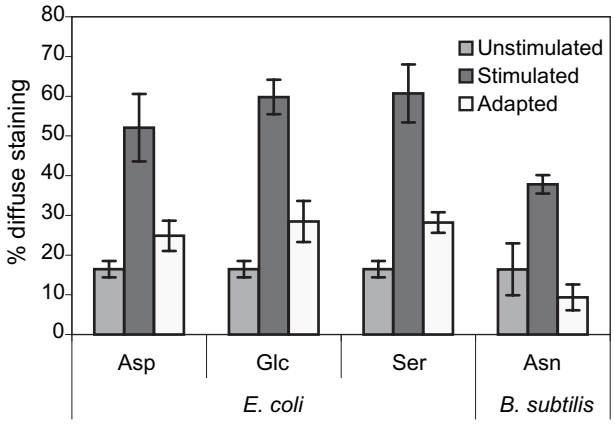

Fig. 1. Percentages of *E. coli* or *B. subtilis* cells exhibiting diffuse staining when treated with chemoattractants at high concentration (1 mM). This concentration is estimated to correspond to 95% receptor occupancy. Adapted cells were pre-treated with 1 mM stimuli.

The MCP lattice includes receptors that are present in relatively high and low (5- to 10-fold less) abundance (Hazelbauer and Engström, 1981; Slocum and Parkinson, 1983; Li and Hazelbauer, 2004). We sought to assess the effects of chemoattractants that initiate signalling through high- and low-abundance receptors. A large increase in the concentration of an attractant (Asp or Ser) that acts through a high-abundance receptor (e.g. Tar or Tsr respectively) or one (glucose) that acts through a low-abundance receptor (Trg) afforded a similar increases in cells exhibiting diffuse MCP staining (Fig. 1). Thus, the observed changes in chemoreceptor proximity can be triggered by signals initiated through either high- or low-abundance MCPs.

Our initial results in *E. coli* prompted us to conduct comparable experiments with *B. subtilis*. We envisioned that these latter experiments would reveal whether the attractant-promoted changes in chemoreceptor localization were conserved across bacterial species. As when *E. coli* were treated with a high attractant concentration, we found the proportion of treated *B. subtilis* cells displaying diffuse staining rose; an increase from 16% to 38% was observed (Fig. 1). Upon adaptation, the number of cells exhibiting diffuse staining returned to pre-stimulus levels. Thus, similar decreases in MCP proximity are elicited by a large increase in attractant concentration in either *B. subtilis* or *E. coli* cells. These data suggest that a large increase in attractant concentration disrupts chemoreceptor localization.

Substantial signal amplification is required under conditions in which less than 2% of the receptors are occupied by attractant. We would expect that MCP–MCP interactions would be required to amplify the signal under these circumstances (Lybarger and Maddock, 2000; Ames *et al*., 2002; Gestwicki and Kiessling, 2002; Lamanna *et al*., 2002; Sourjik and Berg, 2002; Zimmer *et al*., 2002). If disrupting chemoreceptor localization is important for decreasing the sensitivity of the cell to high concentrations of attractants, we would predict that chemoreceptor localization would be maintained at low attractant concentrations. To test this hypothesis, we treated *E. coli* and *B. subtilis* with a low concentration of attractant (1 μM Asn or 0.01 μM Asp) and visualized MCP localization subsequent to paraformaldehyde-mediated cross-linking. In either *E. coli* or *B. subtilis*, no changes in MCP localization were detected relative to untreated cells (Fig. 2).

Repellents induce bacteria to tumble, as they elicit CW flagellar rotation. We tested whether repellents, like attrac-

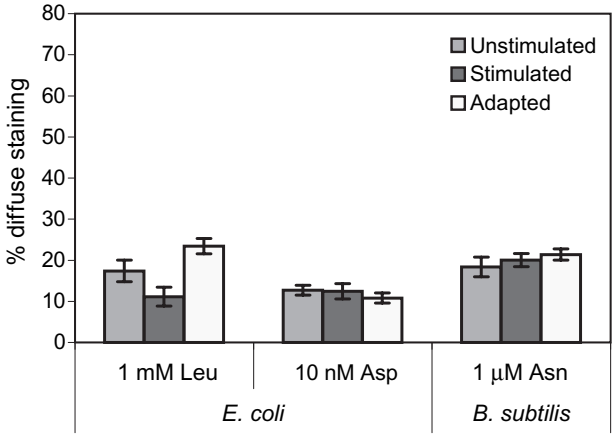

Fig. 2. Percentages of *E. coli* or *B. subtilis* cells exhibiting diffuse staining when treated with a high concentration of repellent (leucine) or a low concentration of attractant (predicted receptor occupancy of less than 2%). The stimulant concentrations were: 1 mM leucine, 10 nM aspartate and 1 μM asparagine. Adapted cells were pre-treated with stimuli at the same concentrations.

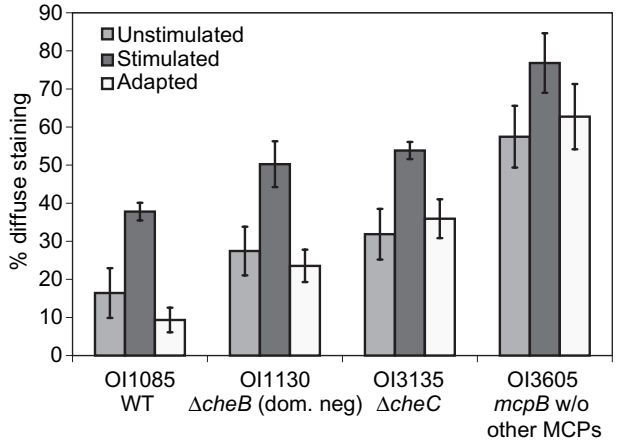

Fig. 3. Percentages of cells exhibiting diffuse staining in *B. subtilis* CheB, CheC and MCP mutant strains. Bacteria were treated with 1 mM asparagine. Adapted cells were pre-treated with 1 mM asparagine.

tants, would alter MCP localization. The addition of repellents did not increase the percentage of cells exhibiting diffuse staining patterns in *E. coli* (Fig. 2). In *E. coli*, repellents may subtly increase the polar localization of the MCPs; this observation suggests that repellents have the inverse effect of attractants on chemoreceptor localization. The effect of repellents on polar staining was modest, however, and definitive conclusions cannot be drawn.

Effects of mutations in signalling components on MCP proximity

We sought to determine whether the observed changes in chemoreceptor localization relate to signal output. To this end, we examined MCP localization in *B. subtilis* strains in which chemotaxis proteins have been modified or deleted. We probed strains that had severe defects in chemotaxis as well as those whose deficiencies were more modest.

To explore the importance of MCP–MCP interactions, we examined *B. subtilis* mutants that produce McpB but not heterologous MCPs. These cells have a low CCW bias (Zimmer *et al*., 2002); they tend to tumble more in the absence of attractant yet respond normally to the addition of attractant. They also adapt normally, although the removal of attractant returns the bacteria to a hyper-tumbly state (Zimmer *et al*., 2002). In our studies, these bacteria often appeared elongated, and, although polar staining was not apparent, the 'diffuse' pattern exhibited by 60% of cells (Fig. 3) was more punctuate than the diffuse staining displayed by other mutants. Although the MCP-depleted cell did not have consistent polar arrays, smaller clusters appeared to be spread throughout.

We investigated the importance of the MCP lattice by using cells deficient in key scaffolding proteins. In *B. subtilis*, deletion of both CheW and its homologue CheV results in cells that have lost chemotactic ability (Rosario *et al*., 1994). When MCP localization was examined in the mutant cells, approximately 50% display diffuse staining (Fig. 4). Upon attractant addition, no change in MCP proximity can be detected. Thus, in these mutants that are unable to undergo chemotaxis, no decrease in MCP localization in response to a high attractant concentration is observed.

We also tested mutant strains with less severe defects in chemotaxis that encode defective CheW or CheV proteins (Fig. 4). The *B. subtilis* strain that lacks CheW shows a small change in CCW bias in response to attractants. In comparison with wild-type cells, a higher percentage of cells of this strain exhibit diffuse staining. When subjected

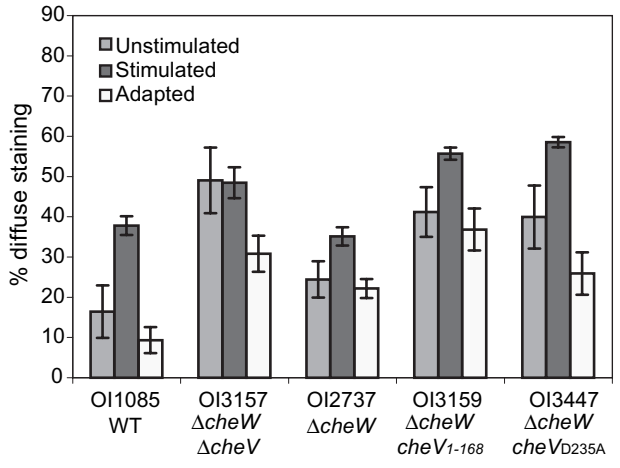

Fig. 4. Percentages of cells exhibiting diffuse staining in *B. subtilis* CheW/CheV mutant strains. Bacteria were treated with 1 mM asparagine. Adapted cells were pre-treated with 1 mM asparagine.

to a large increase in the concentration of Asn attractant (1 mM), only a subtle increase in the percentage of mutant cells exhibiting a diffuse staining pattern is detected. Similarly, we assessed mutants that encode defective CheV variants. CheV has one domain homologous to CheW and a second domain similar to CheY; the latter can be phosphorylated at aspartate 235 (Karatan *et al*., 2001). Cells that cannot produce phospho-CheV, either because they possess a CheV variant in which the domain homologous to CheY is deleted (OI3159) or because an Ala residue replaces the critical Asp (OI3447), exhibit defects in adaptation (Rosario *et al*., 1994; Karatan *et al*., 2001). Their return to pre-stimulus behaviour after exposure to an attractant is partial and occurs after a longer time period. As is seen with wild-type bacteria, however, an increase in the proportion of mutant bacteria exhibiting diffuse staining is elicited by a large boost in stimulant concentration (Fig. 4). As expected for cells that do not properly adapt, the level of polar MCP localization following adaptation does not correspond to that of wild-type cells. The degree of adaptation in the *ΔcheWcheVtrun* is similar to that in the *ΔcheWΔcheV* strain; the degree of adaptation in the *ΔcheWcheV D235A* mutant is greater, although it does not adapt as effectively as wild type. Accordingly, the phenotype of the *ΔcheWcheVtrun* strain is more severe (i.e. it undergoes less adaptation) than of the *ΔcheWcheV D235A* mutant strain (Karatan *et al*., 2001). Thus, for mutant strains that possess minor defects in chemotaxis, the percentage of unstimulated cells exhibiting diffuse staining is increased relative to wild-type cells. As with wild-type cells, however, a large increase in attractant concentration elicits a further increase in the percentage of cells that manifest diffuse staining.

The results obtained for the mutants encoding CheV variants led us to further explore the influence of mutations in the genes critical for adaptation. For example, changes in the methylation state of the chemoreceptor McpB are important for *B. subtilis* adaptation to the chemoattractant asparagine, and the glutamate residue at position 630 has been shown to play a role (Zimmer *et al*., 2000). A mutant that encodes an McpB E630K variant (OI3860) exhibits continuous running behaviour, even in the absence attractant (M. Zimmer and G. Ordal, unpublished results). We postulated that a consistently higher percentage of this mutant would display diffuse staining. The McpB E630K cells exhibited the greatest percentage of diffuse staining. No change was observed upon the addition of chemoattractant (Fig. 5).

We also examined chemoreceptor localization in mutants that show other phenotypes indicative of defects in adaptation. Strains with glutamate-to-aspartate substitutions at key residues for adaptation (McpB E630D and McpB E637D) retain chemotactic ability; however, their ability to adapt is compromised relative to wild-type cells

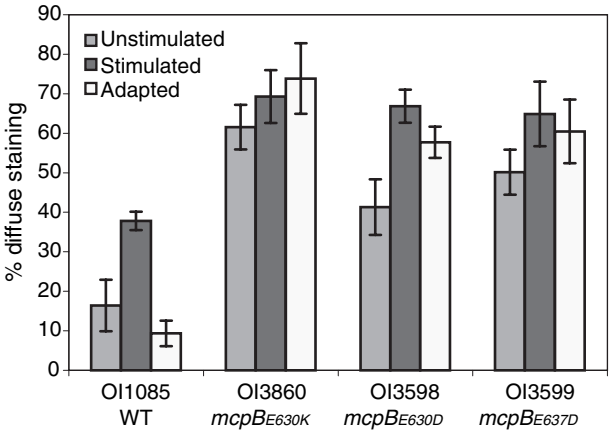

Fig. 5. Percentages of cells exhibiting diffuse staining in *B. subtilis* McpB adaptation mutant strains. Bacteria were treated with 1 mM asparagine. Adapted cells were pre-treated with 1 mM asparagine.

(Zimmer *et al*., 2000). Specifically, the mutant harbouring McpB E630D (OI3598) does not adapt to Asn removal, and the mutant possessing McpB E637D (OI3599) does not adapt to Asn addition. As with the E630K variant, we detected a higher percentage of unstimulated mutant cells with diffuse staining. When an attractant was added at high concentration to these responsive mutant strains, decreases in polar MCP localization resulted. Thus, attractant-induced decreases in the percentage of mutant cells with MCPs localized at the poles are observed for strains that are capable of chemotaxis. The magnitude of the decrease correlates with the mutant's ability to respond properly to attractant.

Cells with defective adaptation responses can also arise from mutations that encode variants of the methyltransferase CheB and the methylesterase CheR. A dominant negative mutation in the gene encoding the methylesterase CheB (OI1130) disrupts the ability of CheA to phosphorylate CheY (Ordal *et al*., 1985); cells with this mutation do not undergo chemotaxis (Ordal *et al*., 1985). Accordingly, we reasoned that signals important for eliciting chemotaxis would be disrupted. The levels of phospho-CheY in these cells should be low, but interactions between the MCPs should be preserved. The percentage of unstimulated mutant OI1130 cells exhibiting diffuse staining was only slightly higher than wild-type cells. As predicted, wild-type-like changes in the percentage of cells exhibiting diffuse MCP localization in response to stimulation and adaptation were observed (Fig. 3).

We also tested whether chemoreceptor localization would be altered in mutants harbouring higher concentrations of phospho-CheY. CheC is a protein that is important for chemotaxis as it acts to promote the hydrolysis of phospho-CheY (Szurmant *et al*., 2004a). A mutant possessing a deletion in *cheC* does not adapt properly to

attractant and shows an increase in post-stimulus CCW bias (Rosario *et al*., 1995). When MCP localization was visualized in this mutant, staining patterns similar to those observed for wild-type *B. subtilis* were obtained (Fig. 3). Thus, changes in the intracellular concentration of the diffusible response regulator CheY result in defective chemotaxis without altering chemoreceptor localization.

Discussion

There is increasing evidence that inter-chemoreceptor interactions are important for signal amplification (Lybarger and Maddock, 2000; Ames *et al*., 2002; Gestwicki and Kiessling, 2002; Lamanna *et al*., 2002; Sourjik and Berg, 2002; 2004; Zimmer *et al*., 2002; Homma *et al*., 2004). Recent reports suggest that different chemoreceptors can signal collaboratively in both *E. coli* and *B. subtilis*, and ligands that can pre-cluster small groups of receptors potentiate subsequent signals through other receptors (Ames *et al*., 2002; Gestwicki and Kiessling, 2002; Lamanna *et al*., 2002; Studdert and Parkinson, 2004). At low attractant levels, increased association between chemoreceptors, that is, stabilization of the MCP lattice, promotes amplification of chemotactic signalling. Conversely, when cells encounter a large increase in attractant concentration, a decrease in the associations in the stability of the MCP array could minimize signal amplification. This scenario provides a means for cells to respond appropriately; signals are amplified only when it is necessary.

We assessed the effect of different stimulants on MCP localization using the lysine-reactive cross-linking agent paraformaldehyde. Using a method such as monitoring the polar localization of green fluorescent protein (GFP)-labelled MCPs, only large and persistent changes in chemoreceptor localization can be detected. Indeed, no changes in MCP localization could be discerned upon the addition of high attractant concentrations to live cells producing GFP–MCP fusion proteins (Homma *et al*., 2004). In contrast, cross-links formed via the reaction of paraformaldehyde occur only between closely associated proteins, and such covalent linkages are relatively stable. Thus, using this reagent, transient changes in receptor proximity can be preserved in the cell for further processing and visualization (Leong, 1994). We reasoned this type of covalent cross-linking reaction would be exquisitely sensitive to changes in receptor proximity.

To determine whether MCP localization is altered under conditions in which signal amplification is not required, we treated *E. coli* or *B. subtilis* cells with high concentrations of attractant (> 95% receptor occupancy), added paraformaldehyde and visualized MCP localization. Upon exposure to this high level of attractant, an increase in the percentage of cells exhibiting diffuse MCP staining was observed for both *B. subtilis* and *E. coli*. We postulate that changes in the fraction of cells exhibiting diffuse staining pattern can be interpreted as a qualitative measure of the stability of the MCP lattice. This diffuse staining might reflect either large changes in MCP localization or subtle alterations in MCP–MCP interactions within the lattice; the latter could be magnified during the processing of samples for visualization. In both cases, we postulate that the change in MCP proximity results from a decrease in the stability of the lattice.

We tested whether changes in MCP localization could be effected by other conditions. When cells were exposed to low attractant concentrations (*c*. 2% receptor occupancy), no change in chemoreceptor proximity could be detected. To further investigate changes in chemoreceptor clustering, we explored the effects of repellents. *E. coli* typically respond more sensitively to attractants than the repellents. Accordingly, the addition of a repellent does not result in measurable changes in the MCP array. When we used high attractant concentrations but performed our experiments after a period of time that would allow for adaptation, the MCPs were localized as in untreated cells. Thus, the chemoreceptors are localized at attractant levels requiring signal amplification, and chemoreceptor localization occurs upon adaptation to reset the system. However, when signal amplification is dispensable, the data indicate that interactions between chemoreceptors are weakened.

Previous theoretical models also have suggested that ligand binding decreases the interactions between MCPs (Bray *et al*., 1998; Albert *et al*., 2004). A recent report (Homma *et al*., 2004) describes data consistent with these models and our results. These studies employed *E. coli* producing engineered Tar variants that possess cysteine residues that can form cross-links via disulphide bond formation. In the presence of a high aspartate concentration (1 mM), the extent of cross-linking between the receptor periplasmic domains decreased and the amount of cross-linked oligomers was reduced. Our data support these predictions: MCP localization is decreased in the presence of a high attractant concentration, a situation in which signal amplification is not required.

Data from mutants implicate a role for MCP localization in controlling output responses. For example, Maddock and co-workers found that high-abundance chemoreceptors, when expressed alone, formed clusters; and *E. coli* producing only a single high-abundance chemoreceptor can perform chemotaxis (Lybarger and Maddock, 2000). In contrast, no clustering was observed in cells producing a single low-abundance MCP. Despite differences in the clustering, both high- and low-abundance receptors were localized at the poles. Still, mutant cells lacking high-abundance MCPs do not exhibit chemotaxis. Using *B. subtilis* mutants that produce McpB but not heterologous

MCPs, we obtained results that also support the importance of chemoreceptor localization. These mutant cells, which respond to attractants but tend to be hypertumbly in the unstimulated state, afforded a punctuate MCP-staining pattern. Thus, the chemoreceptors appear to be localized, although not at the cell poles. Together, these results provide further support for the conclusion that increased chemotactic ability is observed in cells exhibiting higher levels of MCP clustering.

To investigate further the role of receptor clustering in signal amplification, we used a panel of *B. subtilis* mutants that produce variants of key signalling proteins important for chemotactic responses. These defects ranged from dramatic (e.g. mutants that did not respond to chemoattractants) to subtle (e.g. mutants with altered biases in the unstimulated state). For example, we examined mutants with defects in proteins critical for assembling the MCP lattice. In *E. coli* CheW serves as a scaffolding protein that is critical for polar localization of the chemoreceptors; diffuse staining of MCPs is observed for *E. coli* cells in which CheW is non-functional (Maddock and Shapiro, 1993). Moreover, these mutants do not exhibit chemotaxis. *B. subtilis* produce CheW and CheV. Because the latter has an N-terminal CheW-like domain, CheW and CheV are likely to have overlapping functional roles in the assembly of the MCP lattice (Rosario *et al*., 1994; Karatan *et al*., 2001). Consistent with this view, the *cheV* or *cheW* null mutants are capable of chemotaxis but the *cheVcheW* null mutant is not. Accordingly, we probed MCP localization in a *B. subtilis cheVcheW* null mutant. In comparison with wild-type cells, a greater percentage of the mutant cells (*c*. 50%) exhibit diffuse chemoreceptor staining patterns. Interestingly, no change in MCP proximity upon attractant stimulation is observed. This result further implicates a role for receptor proximity in chemotactic signal processing.

The chemotactic responses of *B. subtilis* mutants lacking functional CheW are more nuanced; thus, we examined MCP localization in this background. *B. subtilis* mutants lacking CheW respond to attractants but decreases in chemotactic activity relative to wild-type cells are observed (Rosario *et al*., 1994; Karatan *et al*., 2001). Moreover, a higher percentage of these mutant cells exhibit diffuse MCP staining patterns. Still, we did observe an increase in the percentage of cells displaying diffuse staining upon exposure to a high concentration of attractant, although the changes were subtler than those in wild-type cells. The mutant, which retains its ability to respond to attractants, also exhibits decreases in MCP localization upon stimulation with a high attractant concentration.

Changes in protein phosphorylation are critical for proper chemotactic signal processing within the cell; therefore, we examined *B. subtilis* mutants lacking a phosphorylated response regulator, phospho-CheV. In addition to its N-terminal CheW-like domain, CheV has a C-terminal response regulator domain, and Asp235 of CheV can undergo phosphorylation (Karatan *et al*., 2001). Cells that encode a CheV variant in which the C-terminal domain has been deleted or cells that encode a CheV variant that cannot undergo phosphorylation (D235A) do respond to attractants but cannot adapt properly (Karatan *et al*., 2001). From our studies of MCP localization in wild-type *B. subtilis*, we predicted that the percentage of mutant cells that exhibit diffuse MCP staining would increase after exposure to high concentrations of attractant. As expected for cells capable of responding to attractants, this result, which is similar to that seen for wild-type cells, was obtained. In contrast to the data obtained with wild-type cells, an increase in cells with localized MCP staining after time periods sufficient for adaptation was not observed for the mutant. These data reinforce our findings that reformation of a localized MCP lattice occurs upon adaptation.

To probe further the relationship between MCP localization and adaptation, we used mutant cells encoding chemoreceptors possessing side-chain substitutions at key methylation sites. The most dramatic effects were observed with a mutant encoding an McpB E630K variant that exhibits continual running behaviour. In these cells, the MCP lattice appears to be destabilized dramatically even in the absence of high attractant concentrations. No change in MCP localization is observed upon attractant addition. It is interesting that diffuse chemoreceptor staining patterns are observed for this as well as for the *cheV-cheW* null mutant, as neither strain is capable of chemotaxis.

The hypothesis that the MCP lattice is destabilized at high attractant levels in *B. subtilis* is supported by recent data reported by Bunn and Ordal (2003; 2004). They found that, upon stimulation of the receptors by high concentrations of attractant, the chemoreceptors are more susceptible to CheB methylesterase activity for a short period. A brief destabilization of the MCP lattice upon stimulation could allow CheB to access more readily the cytoplasmic domains of the MCPs. Controlling the accessibility of the chemoreceptors to the methylesterase is another potential role for inter-chemoreceptor communication in modulating chemotactic signalling.

As with wild-type *B. subtilis*, Bunn and Ordal (2004) found that the brief period of increased receptor susceptibility towards the methylesterase occurs in the *cheWcheV* null mutant. The methylesterase activity in the wild-type and mutant bacteria returns to background levels after a similar time period. Disulphide cross-linking experiments indicate that the conformation of the receptors after stimulation, in both wild type and the *cheWcheV* null mutant, differs from that before stimulation (Szurmant *et al*., 2004b). The modest decrease in the percentage of

cells exhibiting diffuse chemoreceptor staining in the *cheWcheV* null mutant (Fig. 4) is consistent with data from the cross-linking experiments.

Our finding that high attractant doses elicit similar changes in receptor proximity in *B. subtilis* and *E. coli* indicates that this response is fundamental and conserved in evolution. Although attractant binding leads to increased CheA phosphorylation in *B. subtilis* and decreased CheA phosphorylation in *E. coli*, decreased MCP localization is observed upon treatment with high levels of attractant in both species. Thus, the observed changes in MCP localization do not depend on the phosphorylation state of CheA. This interpretation is supported by observations that the polar localization of the MCPs is independent of CheA phosphorylation (Skidmore *et al*., 2000) and our data from *B. subtilis* mutants indicating that MCP localization is decoupled from CheY phosphorylation state. Together, the data suggest that there is more flexibility in the mechanism by which the kinase CheA transmits the consequences of receptor occupation than in the effects of attractants on the receptors. Our results suggest that the changes in chemoreceptor conformation and interactions that occur upon attractant binding are conserved in evolution.

A model for understanding the data presented herein builds on previous theoretical constructs that suggest that ligand binding decreases the interactions between MCPs (Bray *et al*., 1998; Albert *et al*., 2004). We propose that attractant-occupied receptors have a higher affinity for unoccupied receptors than for other occupied receptors. Our data are consistent with a scenario in which receptors bound to attractant interact weakly or not at all with other bound receptors. Accordingly, ligand binding could disrupt either a trimer of dimeric MCPs or a higher-order assembly (*vide infra*). We hypothesize that, when unoccupied, receptors associate to form small oligomers that can assemble into an extended array. When chemoattractant binds, the MCP complexes shift to a more open assembly in which the interactions between MCP oligomers in the lattice are destabilized. When high concentrations of attractant are added and signal does not need to be amplified, many contacts between MCPs could be destabilized. This high level of receptor occupation would therefore afford a fluid or loosely associated collection of receptors. This model is supported by our observation that MCP localization decreases after treatment with high attractant concentrations. At low attractant concentrations when signal needs to be greatly amplified, bound MCPs should be rare and therefore less likely to be in proximity to one another. Under these circumstances, we envision that unoccupied receptors would remain associated with occupied receptors to allow for magnification of signal. Methylation and adaptation could reset the system such that the lattice is restored to a 'closed' assembly.

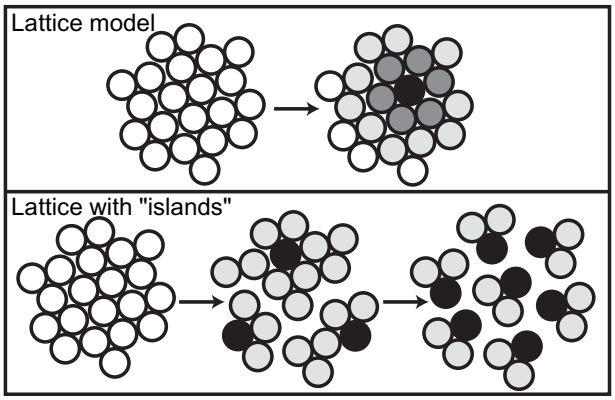

Fig. 6. Model for stimuli-induced changes in lattice organization. Amplification of a chemotactic signal has been described as propagating through a lattice (top). Black circles represent bound ligand, and the signal emitted from an MCP is represented by the intensity of shading of the circle. Our results suggest that the lattice stability changes with respect to ligand concentration. Specifically, we propose that stimuli trigger the dissociation of receptors into 'islands' [bottom; similar to what Parkinson and co-workers (Ames *et al*., 2002) referred to as teams or squads]. The size of the 'islands' is likely proportional to both ligand concentration and signal output.

In our model, MCP oligomers (perhaps trimers of dimers or other oligomers) exist in an extended lattice. Upon chemoattractant binding, proportional destabilization of the lattice would occur; MCP oligomers, perhaps with only one occupied receptor, would dissociate into islands whose size could be related to the extent of signal amplification required (Fig. 6). Larger islands would result in larger signal amplification. Upon adaptation, the MCP oligomers would coalesce and reform the stable array.

One analogy for such a model of signal amplification is found in eukaryotic signalling processes that involve detergent-resistant lipid microdomains. Lipid microdomains can fluctuate in size and change their composition in response to the initiation of signalling pathways (Edidin, 1997). Signalling from the B cell and T cell antigen receptors and the immunoglobulin E receptor, for example, is suggested to occur in dynamic lipid microdomains (Field *et al*., 1997; Cheng *et al*., 1999; Giurisato *et al*., 2003). In these signalling pathways, there is evidence that antigen binding influences the associations between receptor complexes. In B and T cell receptor signalling, it has been proposed that ligand binding shifts the oligomeric receptor complex from a 'closed' to a more 'open' formation (Reth *et al*., 2000; Reth, 2001). Our data suggest that oligomeric MCP complexes may function in a similar way within the chemoreceptor lattice.

There is growing support for the importance of MCP–MCP interactions in chemotactic signal amplification. We have shown that the addition of a high concentration of chemoattractant elicits a decrease in MCP localization in both *E. coli* and *B. subtilis*. The data suggest that high attractant concentrations destabilize the chemoreceptor

Table 1. *Bacillus subtilis* strains and counterclockwise (CCW) biases at high attractant concentration.

Strain	Characteristic	Pre-stimulus CCW bias	Post-stimulus CCW bias	Ratio	References
OI1085	Wild type	56	57	0.99	Rosario *et al*. (1994); Karatan *et al*. (2001)
OI2737	Δ*cheW*	67	64	1.04	Rosario *et al*. (1994); Karatan *et al*. (2001)
HB4007	Δ*cheW*/Δ*cheV*	10	10	–	Rosario *et al*. (1994)
OI3159	Δ*cheW*/*cheV*$_{1\text{-}168}$	65	90	0.72	Rosario *et al*. (1994); Karatan *et al*. (2001)
OI3447	Δ*cheW*/*cheV*$_{D235A}$	60	78	0.77	Karatan *et al*. (2001)
OI3598	*mcpB*$_{E630D}$	40	57	0.70	Zimmer *et al*. (2000)
OI3599	*mcpB*$_{E637D}$	67	89	0.75	Zimmer *et al*. (2000)
OI3860	*mcpB*$_{E630K}$	90	90	–	M. Zimmer and G. Ordal (unpublished)
OI3605	Δ*mcps*, *mcpB*	20	60	0.33	Zimmer *et al*. (2002)
OI1130	Δ*cheB* (dom neg)	0	0	–	Ordal *et al*. (1985)
OI3135	Δ*cheC*	60	78	0.77	Rosario *et al*. (1995)

lattice and that this destabilization corresponds to decreased chemotactic signalling. The evidence presented is consistent with a model in which MCPs exist in oligomers that dynamically dissociate and re-associate. Our data suggest further parallels in the mode of signal modulation in bacteria and eukaryotes.

Experimental procedures

Strains and growth media

Bacillus subtilis strains are listed with references in Table 1. *E. coli* (AW405, J. Adler) were grown in Luria–Bertani medium (1% Tryptone, 0.5% yeast extract, 0.5% NaCl). *B. subtilis* were grown in 1% Tryptone, 0.5% NaCl, 0.2 mM $MgCl_2$, 0.01 mM $MnCl_2$, 10 mM glucose and 0.5% glycerol. All chemicals were reagent grade and obtained from Sigma (St. Louis, MO) unless otherwise noted.

Fluorescence microscopy

Bacteria were prepared as described previously (Gestwicki *et al*., 2000; Lamanna *et al*., 2002). Briefly, bacteria were harvested and washed in phosphate-buffered saline, fixed with 2% paraformaldehyde and allowed to adhere to poly lysine-treated coverslips. *E. coli* were stained with an antibody (1:667) raised to the cytoplasmic portion of Trg (G. Hazelbauer), and *B. subtilis* were stained with an antibody (1:667) raised to McpB (G. Ordal). These antibodies have been shown to label MCPs specifically in *E. coli* (Gestwicki *et al*., 2000; Lamanna *et al*., 2002) and *B. subtilis* (Kirby *et al*., 2000). MCPs were visualized with a goat anti-rabbit secondary antibody labelled with fluorescein (Molecular Probes, Eugene, OR) using a Nikon microscope with the 60× oil-immersion objective and MetaMorph Imaging System software (Universal Imaging Corporation, Downingtown, PA).

The localization of the MCPs in each bacterium was determined by two independent methods. Using the first strategy, bacteria were scored as having polar or diffuse localization of MCPs (Fig. 7) and counted separately by two researchers. Bacteria were assessed in a blinded manner, as experiments were only numbered and those counting the bacteria did not know which number corresponded to which experiment. MCP localization was deduced by comparing the intensity of staining at the poles to that along the lateral edges of the bacterium. Three random fields were counted for each experiment, and each experiment was compared with a wild-type control to ascertain any day-to-day variability. Samples were not normalized because the wild-type controls were consistent in each experiment. A sample data field is provided in Fig. S1 in *Supplementary material*. Each data set was derived from at least three independent experiments. The total number of cells counted ranged from 300 to 700 for each condition. Standard error is reported. In the second method, bacteria were analysed by measuring the fluorescence intensity along a longitudinal cross-section using the ImageJ version 1.32 software package (National Institutes of Health, Bethesda, MD) (Fig. S2). The variation in intensity across the bacterium was measured by calculating the standard deviation from the average of the intensities between, and including, the poles of the bacterium. This standard deviation was scaled to the maximum fluorescence intensity for each bacterium to determine a per cent deviation. Bacteria with a scaled standard deviation greater than 11% were considered to have polar MCP localization. Each method resulted in similar values that were within error (2–3%). Examples are included in Figs S1 and S2 in *Supplementary material*.

Stimulation and adaptation conditions

Stimulants (asparagine, aspartate, serine, glucose or leucine) were added immediately before fixation. Stimuli were used at 1 mM for high concentration experiments. For low concentrations, asparagine was used at 1 μM and aspartate

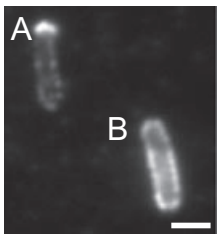

Fig. 7. Example of polar (A) and diffuse (B) staining patterns of *B. subtilis* MCPs. Cells were treated with paraformaldehyde to cross-link proteins then stained with an antibody to the cytoplasmic portion of McpB and a fluorescein-labelled secondary antibody. The bar represents 1 μm.

at 10 nM. For experiments in which adaptation to stimuli was performed, the same concentration of ligand was added to the wash buffer, and bacteria were allowed to adapt to the stimulant for 5 min before paraformaldehyde treatment.

'High' concentrations represent receptor occupancies of 95% or greater, while 'low' concentrations correspond to a receptor occupancy of less than 2%. Receptor occupancy (RO) for each ligand (L) was calculated as follows based on reported dissociation constants (K_d):%RO = (100%)[L]/(K_d + [L]) (Mesibov *et al*., 1973). For example, the K_d for Asn in *B. subtilis* is 56 μM (Ordal *et al*., 1977; Kirby *et al*., 2000), which yields 95% RO at 1 mM. In *E. coli*, the reported dissociation constants for aspartate, glucose and serine are 1.2 μM, 0.6 μM and approximately 50 μM respectively (Mesibov and Adler, 1972; Adler *et al*., 1973; Mesibov *et al*., 1973; Biemann and Koshland, 1994).

Acknowledgements

We thank Professor C. Wiese for use of microscope, S. Bennett for assistance with collecting and scoring bacteria, Professors J. Adler for *E. coli* and G. Hazelbauer for anti-MCP antibody. These studies were supported by the NIH (GM55984 to L.L.K.). A.C.L. was supported by NIH Molecular Biosciences Training Grant (GM072125) and an NSF predoctoral fellowship.

References

Adler, J., Hazelbauer, G.L., and Dahl, M.M. (1973) Chemotaxis towards sugars in *Escherichia coli*. *J Bacteriol* **115:** 824–847.

Alam, M., and Hazelbauer, G.L. (1991) Structural features of methyl-accepting taxis proteins conserved between *Archaebacteria* and *Eubacteria* revealed by antigenic cross-reaction. *J Bacteriol* **173:** 5837–5842.

Albert, R., Chiu, Y.W., and Othmer, H.G. (2004) Dynamic receptor team formation can explain the high signal transduction gain in *Escherichia coli*. *Biophys J* **86:** 2650–2659.

Ames, P., Studdert, C.A., Reiser, R.H., and Parkinson, J.S. (2002) Collaborative signaling by mixed chemoreceptor teams in *Escherichia coli*. *Proc Natl Acad Sci USA* **99:** 7060–7065.

Armitage, J.P., and Schmitt, R. (1997) Bacterial chemotaxis: *Rhodobacter sphaeroides* and *Sinorhizobium meliloti* – variations on a theme? *Microbiology* **143:** 3671–3682.

Barak, R., and Eisenbach, M. (1992) Correlation between phosphorylation of the chemotaxis protein CheY and its activity at the flagellar motor. *Biochemistry* **31:** 1822–1826.

Biemann, H.P., and Koshland, D.E. (1994) Aspartate receptors of *Escherichia coli* and *Salmonella typhimurium* bind ligand with negative and half-of-the-sites cooperativity. *Biochemistry* **33:** 629–634.

Bischoff, D.S., and Ordal, G.W. (1991) Sequence and characterization of *Bacillus subtilis* CheB, a homolog of *Escherichia coli* CheY, and its role in a difference mechanism of chemotaxis. *J Biol Chem* **266:** 12301–12305.

Bischoff, D.S., and Ordal, G.W. (1992) *Bacillus subtilis* chemotaxis: a deviation from the *Escherichia coli* paradigm. *Mol Microbiol* **6:** 23–28.

Bourret, R.B., Borkovich, K.A., and Simon, M.I. (1991) Signal transduction pathways involving protein phosphorylation in prokaryotes. *Annu Rev Biochem* **60:** 401–441.

Bray, D. (2002) Bacterial chemotaxis and the question of gain. *Proc Natl Acad Sci USA* **99:** 7–9.

Bray, D., Levin, M.D., and Morton-Firth, C.J. (1998) Receptor clustering as a cellular mechanism to control sensitivity. *Nature* **393:** 85–88.

Bunn, M.W., and Ordal, G.W. (2003) Transmembrane organization of the *Bacillus subtilis* chemoreceptor McpB deduced by cysteine disulfide crosslinking. *J Mol Biol* **331:** 941–949.

Bunn, M.W., and Ordal, G.W. (2004) Receptor conformation changes enhance methylesterase activity during chemotaxis by *Bacillus subtilis*. *Mol Microbiol* **51:** 721–728.

Burgess-Cassler, A., Ullah, A.H., and Ordal, G.W. (1982) Purification and characterization of *Bacillus subtilis* methyl-accepting chemotaxis protein methyltransferase II. *J Biol Chem* **257:** 8412–8417.

Cheng, P.C., Dykstra, M.L., Mitchell, R.N., and Pierce, S.K. (1999) A role for lipid rafts in B cell antigen receptor signaling and antigen targeting. *J Exp Med* **190:** 1549–1560.

Duke, T.A.J., and Bray, D. (1999) Heightened sensitivity of a lattice of membrane receptors. *Proc Natl Acad Sci USA* **96:** 10104–10108.

Edidin, M. (1997) Lipid microdomains in cell surface membranes. *Curr Opin Struct Biol* **7:** 528–532.

Falke, J.J., and Koshland, D.E., Jr (1987) Global flexibility in a sensory receptor: a site-directed crosslinking approach. *Science* **237:** 1596–1600.

Field, K.A., Holowka, D., and Baird, B. (1997) Compartmentalized activation of the high affinity immunoglobulin E receptor within membrane domains. *J Biol Chem* **272:** 4276–4280.

Fredrick, K.L., and Helmann, J.D. (1994) Dual chemotaxis signaling pathways in *Bacillus subtilis*: a σ^D-dependent gene encodes a novel protein with both CheW and CheY homologous domains. *J Bacteriol* **176:** 2727–2735.

Fuhrer, D.K., and Ordal, G.W. (1991) *Bacillus subtilis* CheN, a homolog of CheA, the central regulator of chemotaxis in *Escherichia coli*. *J Bacteriol* **173:** 7443–7448.

Garrity, L.F., and Ordal, G.W. (1997) Activation of the CheA kinase by asparagine in *Bacillus subtilis* chemotaxis. *Microbiology* **143:** 2945–2951.

Gegner, J.A., Graham, D.R., Roth, A.F., and Dahlquist, F.W. (1992) Assembly of an MCP receptor, CheW, and kinase CheA complex in the bacterial chemotaxis signal transduction pathway. *Cell* **70:** 975–982.

Gestwicki, J.E., and Kiessling, L.L. (2002) Inter-receptor communication through arrays of bacterial chemoreceptors. *Nature* **415:** 81–84.

Gestwicki, J.E., Lamanna, A.C., Harshey, R.M., McCarter, L.L., Kiessling, L.L., and Adler, J. (2000) Evolutionary conservation of methyl-accepting chemotaxis protein location in *Bacteria* and *Archaea*. *J Bacteriol* **182:** 6499–6502.

Giurisato, E., McIntosh, D.P., Tassi, M., Gamberucci, A., and Benedetti, A. (2003) T cell receptor can be recruited to a subset of plasma membrane rafts, independently of cell signaling and attendantly to raft clustering. *J Biol Chem* **278:** 6771–6778.

Goldman, D.J., Worobec, S.W., Siegel, R.B., Hecker, R.V.,

and Ordal, G.W. (1982) Chemotaxis in *Bacillus subtilis*: effects of attractants on the level of methylation of methyl-accepting chemotaxis proteins and the role of demethylation in the adaptation process. *Biochemistry* **21:** 915–920.

Hanlon, D.W., Márquez-Magaña, L.M., Carpenter, P.B., Chamberlin, M.J., and Ordal, G.W. (1992a) Sequence and characterization of *Bacillus subtilis* CheW. *J Biol Chem* **267:** 12055–12060.

Hanlon, D.W., Carpenter, P.B., and Ordal, G.W. (1992b) Influence of attractants and repellents on methyl group turnover on methyl-accepting chemotaxis proteins of *Bacillus subtilis* and role of CheW. *J Bacteriol* **174:** 4218–4222.

Harrison, D., Skidmore, J., Armitage, J., and Maddock, J. (1999) Localization and environmental regulation of MCP-like proteins in *Rhodobacter sphaeroides*. *Mol Microbiol* **31:** 885–892.

Hazelbauer, G.L., and Engström, P. (1981) Multiple forms of methyl-accepting chemotaxis proteins distinguished by a factor in addition to multiple methylation. *J Bacteriol* **145:** 35–42.

Homma, M., Shiomi, D., Homma, M., and Kawagishi, I. (2004) Attractant binding alters arrangement of chemoreceptor dimers within its cluster at a cell pole. *Proc Natl Acad Sci USA* **101:** 3462–3467.

Hughson, A.G., and Hazelbauer, G.L. (1996) Detecting the conformational change of transmembrane signaling in a bacterial chemoreceptor by measuring effects on disulfide cross-linking *in vivo*. *Proc Natl Acad Sci USA* **93:** 11546–11551.

Jasuja, R., Lin, Y., Trentham, D.R., and Khan, S. (1999) Response tuning in bacterial chemotaxis. *Proc Natl Acad Sci USA* **96:** 11346–11351.

Karatan, E., Saulmon, M.M., Bunn, M.W., and Ordal, G.W. (2001) Phosphorylation of the response regulator CheV is required for adaptation to attractants during *Bacillus subtilis* chemotaxis. *J Biol Chem* **276:** 43618–43626.

Kim, K.K., Yokota, H., and Kim, S.-H. (1999) Four-helical bundle structure of the cytoplasmic domain of a serine chemotaxis receptor. *Nature* **400:** 787–792.

Kim, C., Jackson, M., Lux, R., and Khan, S. (2001) Determinants of chemotactic signal amplification in *Escherichia coli*. *J Mol Biol* **307:** 119–135.

Kim, S.-H., Wang, W., and Kim, K.K. (2002) Dynamic and clustering model of bacterial chemotaxis receptors: structural basis for signaling and high sensitivity. *Proc Natl Acad Sci USA* **99:** 11611–11615.

Kirby, J.R., Niewold, T.B., Maloy, S., and Ordal, G.W. (2000) CheB is required for behavioral responses to negative stimuli during chemotaxis in *Bacillus subtilis*. *Mol Microbiol* **35:** 44–57.

Lamanna, A.C., Gestwicki, J.E., Strong, L.E., Borchardt, S.L., Owen, R.M., and Kiessling, L.L. (2002) Conserved amplification of chemotactic responses through chemoreceptor interactions. *J Bacteriol* **184:** 4981–4987.

Lee, G.F., Lebert, M.R., Lilly, A.A., and Hazelbauer, G.L. (1995) Transmembrane signaling characterized in bacterial chemoreceptors using sulfhydryl cross-linking *in vivo*. *Proc Natl Acad Sci USA* **92:** 3391–3395.

Leong, A.S.-Y. (1994) Fixation and fixatives. In *Laboratory Histopathology: A Complete Reference*, Vol. 4. Woods, E.A., and Ellis, R.C. (eds). Edinburgh: Churchill Livingstone, pp. 1–26.

Levit, M.N., Grebe, T.W., and Stock, J.B. (2002) Organization of the receptor-kinase signaling array that regulates *Escherichia coli* chemotaxis. *J Biol Chem* **277:** 36748–36754.

Li, M.S., and Hazelbauer, G.L. (2004) Cellular stoichiometry of the components of the chemotaxis signaling complex. *J Bacteriol* **186:** 3687–3694.

Lybarger, S.R., and Maddock, J.R. (2000) Differences in the polar clustering of the high- and low-abundance chemoreceptors of *Escherichia coli*. *Proc Natl Acad Sci USA* **97:** 8057–8062.

Maddock, J.R., and Shapiro, L. (1993) Polar location of the chemoreceptor complex in the *Escherichia coli* cell. *Science* **259:** 1717–1723.

Mello, B.A., and Tu, Y. (2003) Quantitative modeling of sensitivity in bacterial chemotaxis: the role of coupling among different chemoreceptor species. *Proc Natl Acad Sci USA* **100:** 8223–8228.

Mesibov, R., and Adler, J. (1972) Chemotaxis toward amino acids in *Escherichia coli*. *J Bacteriol* **112:** 315–326.

Mesibov, R., Ordal, G.W., and Adler, J. (1973) The range of attractant concentrations for bacterial chemotaxis and the threshold and size over this range. *J Gen Physiol* **62:** 203–223.

Milligan, D.L., and Koshland, D.E., Jr (1988) Site-directed crosslinking. Establishing the dimeric structure of the aspartate receptor of bacterial chemotaxis. *J Biol Chem* **263:** 6268–6275.

Morgan, D.G., Baumgartner, J.W., and Hazelbauer, G.L. (1993) Proteins antigenically related to methyl-accepting chemotaxis proteins of *Escherichia coli* detected in a wide range of bacterial species. *J Bacteriol* **175:** 133–140.

Moual, H.L., and Koshland, D.E., Jr (1996) Molecular evolution of the C-terminal cytoplasmic domain of a superfamily of bacterial receptors involved in taxis. *J Mol Biol* **261:** 568–585.

Ordal, G.W., Villani, D.P., and Gibson, K.J. (1977) Amino acid chemoreceptors of *Bacillus subtilis*. *J Bacteriol* **129:** 156–165.

Ordal, G.W., Parker, H.M., and Kirby, J.R. (1985) Complementation and characterization of chemotaxis mutants of *Bacillus subtilis*. *J Bacteriol* **164:** 802–810.

Parkinson, J.S. (1993) Signal transduction schemes of bacteria. *Cell* **73:** 857–871.

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.

Reth, M. (2001) Oligomeric antigen receptors: a new view on signaling for the selection of lymphocytes. *Trends Immunol* **22:** 356–360.

Reth, M., Wienands, J., and Schamel, W.W.A. (2000) An unsolved problem of the clonal selection theory and the model of an oligomeric B-cell antigen receptor. *Immunol Rev* **176:** 10–18.

Rosario, M.M.L., 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.

Rosario, M.M.L., Kirby, J.R., Bochar, D.A., and Ordal, G.W. (1995) Chemotactic methylation and behavior in *Bacillus subtilis*: role of two unique proteins, CheC and CheD. *Biochemistry* **34:** 3823–3831.

Schuster, S.C., Swanson, R.V., Alex, L.A., Bourret, R.B., and Simon, M.I. (1993) Assembly and function of a quaternary signal transduction complex by surface plasmon resonance. *Nature* **365:** 343–347.

Segall, J.E., Block, S.M., and Berg, H.C. (1986) Temporal comparisons in bacterial chemotaxis. *Proc Natl Acad Sci USA* **83:** 8987–8991.

Shi, Y., and Duke, T. (1998) Cooperative model of bacterial sensing. *Phys Rev E* **58:** 6399–6406.

Skidmore, J.M., Ellefson, D.D., McNamara, B.P., Couto, M.M.P., Wolfe, A.J., and Maddock, J.R. (2000) Polar clustering of the chemoreceptors complex in *Escherichia coli* occurs in the absence of complete CheA function. *J Bacteriol* **182:** 967–973.

Slocum, M.K., and Parkinson, J.S. (1983) Genetics of methyl-accepting chemotaxis proteins in *Escherichia coli*: organization of the *tar* region. *J Bacteriol* **155:** 565–577.

Sourjik, V., and Berg, H.C. (2002) Receptor sensitivity in bacterial chemotaxis. *Proc Natl Acad Sci USA* **99:** 123–127.

Sourjik, V., and Berg, H. (2004) Functional interactions between receptors in bacterial chemotaxis. *Nature* **428:** 437–441.

Springer, W.R., and Koshland, D.E., Jr (1977) Identification of a protein methyltransferase as the *CheR* gene product in the bacterial sensing system. *Proc Natl Acad Sci USA* **74:** 533–537.

Stock, A.M. (1999) A nonlinear stimulus-response relation in bacterial chemotaxis. *Proc Natl Acad Sci USA* **96:** 10945–10947.

Stock, J.B., and Koshland, D.E., Jr (1978) A protein methylesterase involved in bacterial sensing. *Proc Natl Acad Sci USA* **75:** 3659–3663.

Studdert, C.A., and Parkinson, J.S. (2004) Crosslinking snapshots of bacterial chemoreceptor squads. *Proc Natl Acad Sci USA* **101:** 2117–2122.

Szurmant, H., Muff, T.J., and Ordal, G.W. (2004a) *Bacillus subtilis* CheC and FliY are members of a novel class of CheY-P-hydrolyzing proteins in the chemotactic signal transduction cascade. *J Biol Chem* **279:** 21787–21792.

Szurmant, H., Bunn, M.W., Cho, S.H., and Ordal, G.W. (2004b) Ligand-induced conformational changes in the *Bacillus subtilis* chemoreceptor McpB determined by disulfide crosslinking *in vivo*. *J Mol Biol* **344:** 919–928.

Thoelke, M.S., Kirby, J.R., and Ordal, G.W. (1989) Novel methyl transfer during chemotaxis in *Bacillus subtilis*. *Biochemistry* **28:** 5585–5589.

West, A.H., and Stock, A.M. (2001) Histidine kinases and response regulator proteins in two-component signaling systems. *Trends Biochem Sci* **26:** 369–376.

Wylie, D., Stock, A., Wong, C.-Y., and Stock, J. (1988) Sensory transduction in bacterial chemotaxis involves phophotransfer between Che proteins. *Biochem Biophys Res Commun* **151:** 891–896.

Zimmer, M.A., Tiu, J., Collins, M.A., and Ordal, G.W. (2000) Selective methylation changes on the *Bacillus subtilis* chemotaxis receptor McpB promote adaptation. *J Biol Chem* **275:** 24264–24272.

Zimmer, M.A., Szurmant, H., Saulmon, M.M., Collins, M.A., Bant, J.S., and Ordal, G.W. (2002) The role of heterologous receptors in McpB-mediated signaling in *Bacillus subtilis* chemotaxis. *Mol Microbiol* **45:** 555–568.

Supplementary material

The following supplementary material is available for this article online:

Appendix S1. Supplementary material.

Fig. S1. Raw data (one field) of CheW/CheV-deleted *B. subtilis* (HB4007) stained with anti-McpB antibody and a fluorescent secondary antibody collected for analysis of MCP cross-linking.

Fig. S2. A. Line scan analysis of a bacterium displaying polar MCP staining.

B. A similar analysis for a bacterium displaying diffuse MCP staining.