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Site-specific methylation in *Bacillus subtilis* chemotaxis: effect of covalent modifications to the chemotaxis receptor McpB

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The *Bacillus subtilis* chemotaxis pathway employs a receptor methylation system that functions differently from the one in the canonical *Escherichia coli* pathway. Previously, we hypothesized that *B. subtilis* employs a site-specific methylation system for adaptation where methyl groups are added and removed at different sites. This study investigated how covalent modifications to the adaptation region of the chemotaxis receptor McpB altered its apparent affinity for its cognate ligand, asparagine, and also its ability to activate the CheA kinase. This receptor has three closely spaced adaptation sites located at residues Gln371, Glu630 and Glu637. We found that amidation, a putative methylation mimic, of site 371 increased the receptor's apparent affinity for asparagine and its ability to activate the CheA kinase. Conversely, amidation of sites 630 and 637 reduced the receptor's ability to activate the kinase but did not affect the apparent affinity for asparagine, suggesting that activity and sensitivity are independently controlled in *B. subtilis*. We also examined how electrostatic interactions may underlie this behaviour, using homology models. These findings further our understanding of the site-specific methylation system in *B. subtilis* by demonstrating how the modification of specific sites can have varying effects on receptor function.

INTRODUCTION

Peritrichiously flagellated bacteria can swim towards attractants and away from repellents by altering the frequency of smooth runs and reorientating tumbles through a process known as chemotaxis (Rao & Ordal, 2009). At the core of this navigational system is a mechanism for gradient sensing. Bacteria detect gradients by comparing their presently sensed chemical environment with that sensed in the recent past (Berg & Purcell, 1977; Macnab & Koshland, 1972). If the bacterium is travelling in a favourable direction, up a gradient of attractant or down one of repellent, then it will tend to continue along its current trajectory by decreasing the frequency of tumbles (Berg & Brown, 1972). A critical element of this gradient-sensing mechanism is sensory adaptation: the increase in bias (tendency for counter-clockwise rotation of the flagella) caused by addition of attractant does not persist and, despite continued presence of the attractant, the bias returns to its pre-stimulus value. In fact, the bias is proportional to the rate of change in the average number of ligand-bound receptors and not their absolute levels (Segall *et al.*, 1986).

In the soil bacterium *Bacillus subtilis*, the chemotaxis pathway employs a modified two-component system (Rao & Ordal, 2009). Briefly, the transmembrane chemotaxis receptors form complexes with a histidine kinase, CheA, and two coupling proteins, CheW and CheV. Upon addition of attractant, the receptors change their conformation to cause increased levels of phosphorylated CheA kinase (CheAp). The receptors are able to communicate with the flagellar motors as the phosphoryl group on the kinase is transferred to the soluble response regulator, CheY. Phosphorylated CheY (CheYp) binds to the flagellar motors and increases the likelihood of counter-clockwise rotation and runs (Bischoff & Ordal, 1991; Bischoff *et al.*, 1993; Garrity & Ordal, 1997).

B. subtilis employs three parallel systems for sensory adaptation (Rao *et al.*, 2008). One of these involves phosphorylation of CheV, which has both a CheW domain and a CheY domain, by the kinase (Karatan *et al.*, 2001). Phosphorylated CheV is believed to inhibit the coupling between the kinase and the chemotaxis receptors (Rao *et al.*, 2008). Another involves CheC, whose binding of CheD is enhanced by CheYp. When CheYp concentrations are high, CheC recruits CheD away from the receptors to make them less active (Muff & Ordal, 2007).

The third system involves receptor methylation, the focus of this study. In the methylation adaptation system, two

enzymes, CheR and CheB, add and remove methyl groups, respectively, at conserved glutamate residues on the receptors in response to changes in attractant binding (Burgess-Cassler *et al.*, 1982; Goldman *et al.*, 1984). When the receptors bind attractant, they are rapidly demethylated and then slowly remethylated, with the remethylation phase persisting until the degree of receptor methylation returns to its pre-stimulus levels (Kirby *et al.*, 1999). Irrespective of the ambient concentration of attractant, the net level of methylation in *B. subtilis* is roughly constant at steady state (Kirby *et al.*, 1999). Interestingly, this remethylation step takes many times longer than the behavioural adaptation period. The same two-step process also occurs when the attractant is removed: rapid demethylation followed by slow remethylation.

Based on these observations, we previously hypothesized that receptor methylation is site-specific in *B. subtilis*, so that during adaptation to changes in attractant or repellent concentration, methyl groups are added and removed at these different sets of residues (Zimmer *et al.*, 2000). Specifically, we hypothesized that the methylation of some residues activates the kinase whereas the methylation of others inhibits the kinase. In this study, we tested this hypothesis by exploring how covalent modifications to the three adaptation sites of the canonical *B. subtilis* receptor McpB altered the apparent affinity for its cognate ligand, asparagine, and affected kinase activity. Consistent with this model, we found that the modification of Glu371 increased McpB's apparent affinity for asparagine and also, in most cases, increased the activity of the receptor in stimulating the kinase. Modifications of Glu630 and Glu637, on the other hand, decreased the activity of the receptor in stimulating the kinase. Furthermore, we also examined how electrostatic interactions may underlie this behaviour.

METHODS

Bacterial strains and plasmids. All *B. subtilis* strains are derived from the chemotactic strain (Che^+) OI1085 (Ullah & Ordal, 1981). All cloning and plasmid propagation were performed in *Escherichia coli* strain TG1 (GE Healthcare Life Sciences).

The *B. subtilis* strains were created by using long PCR mutagenesis on the pAIN750 plasmid, which contains the full-length *mcpB* receptor gene under control of its native promoter, as previously described (Zimmer *et al.*, 2000, 2002). These mutated receptors were then integrated back into the *amyE* locus of the Δ10*mcp cheB cheR* strain OI3635 (Hou *et al.*, 2000; Kristich & Ordal, 2002), which lacks all ten *B. subtilis* chemoreceptors along with CheR and CheB. Expression levels of McpB were confirmed using Western blots, and found to be similar to wild-type for all mutant alleles tested.

Sensitivity capillary assay. Sensitivity capillary assays were performed as previously described (Mesibov *et al.*, 1973; Ordal *et al.*, 1977). In these assays, each capillary contained 3.16-fold more attractant than the concentration found in the corresponding pond. The experiments were performed over a range of concentrations spanning eight orders of magnitude. The number of bacteria that enter the capillary is proportional to the difference in the number of receptors titrated with attractant at the two end points – the capillary concentration and the pond concentration. The numbers of bacteria per capillary were plotted as a function of the geometric mean of the concentrations of attractant in the capillary and in the pond. The centre of symmetry of the peak in this corresponding graph is the concentration at which chemotaxis is most potent and, hence, provides an apparent dissociation constant for the receptors (Mesibov *et al.*, 1973).

Cells were grown overnight at 30 °C on TBAB [tryptose blood agar: 1 % (w/v) tryptone, 0.3 % (w/v) beef extract, 0.5 % (w/v) NaCl, 1.5 % agar] plates. The cells were then scraped from the plate and resuspended to OD_{600} 0.014 in 40 ml capillary assay minimal medium (50 mM K_3PO_4, pH 7.0, 1.2 mM $MgCl_2$, 0.14 mM $CaCl_2$, 1 mM $(NH_4)_2SO_4$, 0.01 mM $MnCl_2$, 20 mM sorbitol and 0.02 % tryptone, supplemented with 50 mg ml^{-1} each of histidine, methionine and tryptophan). The cultures were grown to OD_{600} 0.4 at 37 °C with shaking, after which a 50 ml aliquot of a 5 % (v/v) glycerol/0.5 M sodium lactate solution was added, and the cells were incubated for an additional 15 min. They were then washed three times with chemotaxis buffer and diluted to OD_{600} 0.001. The cells were supplemented with the appropriate asparagine concentration and aliquoted into 0.3 ml ponds on a temperature-controlled plate at 37 °C. Closed-end capillary tubes filled with the corresponding amount of asparagine were inserted into the pond. After 30 min, the cells in the capillaries were harvested and transferred to 0.5 ml top agar [1 % (w/v) tryptone, 0.8 % (w/v) NaCl, 0.8 % (w/v) agar, 0.5 mM EDTA] and plated onto TB [1 % (w/v) tryptone, 0.5 % (w/v) NaCl] agar plates. These plates were incubated at 37 °C for 16 h, at which point colonies were counted to derive the data. Experiments were performed in triplicate and on two different days to ensure reproducibility. The error for the dissociation constant (K_d) estimates is less than 5 %, as determined by five separate experiments performed on the wild-type strain OI1085.

Tethered cell assay. The tethered cell assay was performed as described previously (Block *et al.*, 1983; Kirby *et al.*, 1999; Saulmon *et al.*, 2004). Bacterial cells were adhered by their flagella to a microscope coverslip by an anti-flagellin antibody, and the rotational direction, clockwise (CW) or counter-clockwise (CCW), was tracked for a period of 5 min. Data averaged over a population of at least 16 cells resulted in a probability, or bias, of CCW rotation (smooth swim).

The cells were grown using the same protocol as in the sensitivity capillary assay. Once the cells were harvested, the flagella were sheared in a Waring blender by two 15 s bursts. The cells were then pelleted and resuspended in fresh chemotaxis buffer [10 mM K_3PO_4, pH 7.0, 0.14 mM $CaCl_2$, 0.3 mM $(NH_4)_2SO_4$, 0.1 mM EDTA, 5 mM sodium lactate, 0.05 % (v/v) glycerol]. They were then applied to a round coverslip previously incubated with anti-flagellin antibodies for 20 min. The coverslip was fixed to a flow cell, and rotation of the cells was observed with a phase-contrast microscope. Chemotaxis buffer supplemented with asparagine was continuously flowed past the cells. Hobson Tracker software (Hobson Tracking Systems) was employed to track the rotational direction of individual cells, and Matlab (The Mathworks) was used to process the resultant data.

Structural modelling and electrostatic analysis. Structural models of the McpB cytoplasmic domain were generated using SPDBV software and the SWISS-MODEL homology server (Arnold *et al.*, 2006; Kiefer *et al.*, 2009). The closely related *Thermatoga maritima* TM1143 receptor crystal structure served as the template (Park *et al.*, 2006). The cytoplasmic domains of McpB and TM1143 share 27 % identity, and the root mean square deviation (RMSD) of the final McpB model when compared with the TM1143 crystal structure was 0.063 Å (0.0063 nm). The solvent-accessible electrostatic surface potential of each model was analysed using APBS (Baker *et al.*, 2001). The potentials were calculated for a protein core dielectric of 2 and a solvent dielectric

constant of 80. The isopotential contours are shown for an electrostatic energy of ±8 *kT*/e.

RESULTS

Behavioural effect of glutamine substitutions

McpB, the sole receptor for chemotaxis towards asparagine (Hanlon & Ordal, 1994), has three adaptation sites located at residues Gln371, Glu630 and Glu637 (Zimmer *et al.*, 2000), confirmed using tandem mass spectroscopy (G. Glekas, unpublished). A structural model of the cytoplasmic domain of McpB, based on the crystal structure of the closely related *T. maritima* chemoreceptor TM1143 (Alexander & Zhulin, 2007; Park *et al.*, 2006), shows the spatial orientation of the three adaptation sites as constituting a triad (Fig. 1). By contrast, the methylation sites in the *E. coli* Tar receptor are arranged in a line (Fig. 1).

To determine how the covalent modification of these three residues affects the sensitivity and activity of the associated kinase, we tested the effect of substituting glutamines and glutamates in all possible combinations at the three adaptation sites of the canonical McpB receptor.

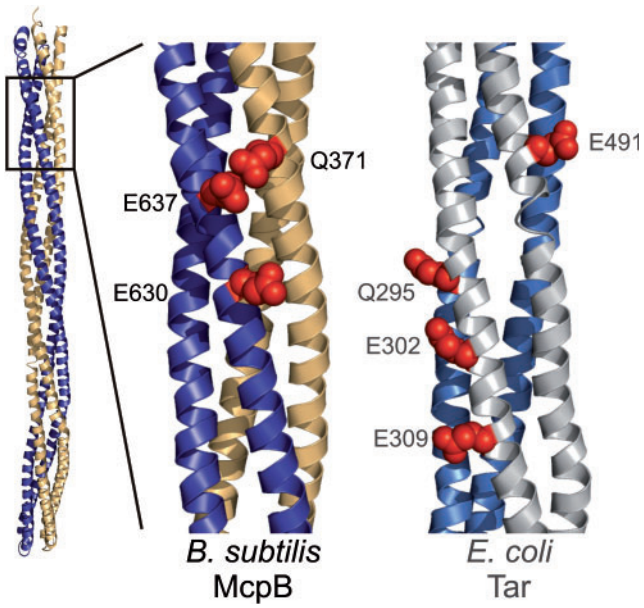

Fig. 1. Structural depictions of the *B. subtilis* McpB and *E. coli* Tar cytoplasmic domains. The homology model shown for McpB is based on the crystal structure of the cytoplasmic domain of the *T. maritima* TM1143 chemoreceptor (Park *et al.*, 2006). The adaptation sites, Gln371, Glu630 and Glu637, form a closely spaced, interacting triad on McpB. In comparison, the *E. coli* Tar cytoplasmic domain shows a different orientation of adaptation sites, all in a line and spaced further apart. Shown is the methylation domain from the structural model for the full receptor (Kim *et al.*, 1999). For easier visualization, the adaptation sites (shown in red) are only highlighted on one face of the receptor.

Substituting glutamates with glutamines has long been used to study the effects of methylation in *E. coli* chemotaxis (Dunten & Koshland, 1991). In particular, the amine replaces the negatively charged carboxylate anion, mimicking the effect of carboxyl methylation.

While the chemotaxis pathways in *B. subtilis* and *E. coli* differ in many ways (Szurmant & Ordal, 2004), we reasoned that the mimicking effect of carboxyl methylation by amidation would be conserved in *B. subtilis*. Our justification is as follows. The structure of the adaptation region, the coiled-coil region of the receptor where the adaptation sites are located, is similar in *E. coli* and *B. subtilis* (Alexander & Zhulin, 2007; Park *et al.*, 2006). The adaptation sites in *B. subtilis* and *E. coli* also share the same heptadic motif (Zimmer *et al.*, 2000). Moreover, CheB and CheR from *B. subtilis* functionally complement the corresponding *E. coli* null mutant (Kirsch *et al.*, 1993a, b), indicating that the recognition sequence and local structure of the receptors is the same, as are the governing reactions for reversible methylation. Finally, the evidence to date strongly indicates that the receptors in virtually all bacteria examined, including *T. maritima*, are organized in semi-ordered hexagonal lattices located predominantly at the cell poles (Briegel *et al.*, 2009). Since the cytoplasmic parts of the receptors in *B. subtilis* and *T. maritima* are very similar (Alexander & Zhulin, 2007), they would probably be organized in similar arrays.

In *E. coli* the amidation, and presumably methylation, of the negatively charged glutamates has previously been shown to neutralize repulsive electrostatic interactions between neighbouring receptors (Starrett & Falke, 2005). Given that the structure of the adaptation region and higher-order organization of the receptors appear to be the same in both *E. coli* and *B. subtilis*, carboxyl methylation probably neutralizes the repulsive effects of the negatively charged glutamates in the *B. subtilis* receptors as well. While we lack definitive evidence, all lines of evidence suggest that amidation will also mimic methylation in *B. subtilis*.

To test the effect of covalent modifications of the adaptation sites the mutated McpB receptors were expressed in a strain lacking the CheR methyltransferase, the CheB methylesterase, the wild-type McpB receptor and the other nine chemotaxis receptors. While CheD, a chemotaxis protein present in *B. subtilis* but not *E. coli*, is also capable of deamidating specific glutamine residues on McpB (Kristich & Ordal, 2002), we have previously found that it did not deamidate residue Gln371 (Kristich, 2002), which presumably gets deamidated by CheB. In addition, we found that CheD does not deamidate engineered glutamines at the adaptation sites (data not shown). To determine the *in vivo* effect of these modifications, we carried out two types of experiments, a sensitivity capillary assay and a tethered cell assay.

Sensitivity capillary assay to assess 'apparent K_d'

In the sensitivity capillary assay, the traditional capillary assay is altered such that a wide range of attractant

concentrations is spanned by 3.16-fold gradients, where the capillary concentration is 3.16 times that in the pond (bacterial suspension). Bacteria will swim up into the capillary, and the number that do so is proportional to the number of receptors titrated at the upper concentration minus the number titrated at the lower concentration. From a graph of the results, the 'apparent K_d' can be determined (Mesibov *et al.*, 1973). This value is the concentration where chemotaxis is maximal, namely the concentration at which the receptors are half bound with attractant and half free. The numerical value reflects not only the intrinsic K_d of the receptor for the attractant but also constraints on receptor conformation and signalling caused by the binding of other chemotaxis proteins, including CheA, CheD, CheW and CheV.

One great value of the experiments performed here is that they were carried out on a static methylation system in which various combinations of glutamines and glutamates at each of the three methylation sites were assessed without the possibility of their being altered during the course of the experiment. However, since chemotaxis requires both excitation and adaptation, this experimental set-up would not be possible in many bacteria. But, in *B. subtilis*, there are two other functional adaptation systems besides the methylation system to facilitate taxis.

When sensitivity capillary assays were carried out on the various mutants, it was discovered that the 'apparent K_d' (as defined above) was 10-fold to 20-fold higher (less apparent affinity) when there was a glutamate at site 371 than when a glutamine was at this site (results shown in Fig. 2 and summarized in Table 1). However, the presence of a glutamine or glutamate at sites 630 or 637 did not much affect the 'apparent K_d'. These results show that the modification state of residue 371 affects McpB's sensitivity towards asparagine.

There were two instances, however, when both sites 630 and 637 were encoded as glutamines, that chemotaxis was too feeble for the 'apparent K_d' to be measured, regardless of the allelic state of site 371 (371E/630Q/637Q and 371Q/630Q/637Q). Interestingly, as outlined below, these are the states where kinase activity would be expected to be the most reduced.

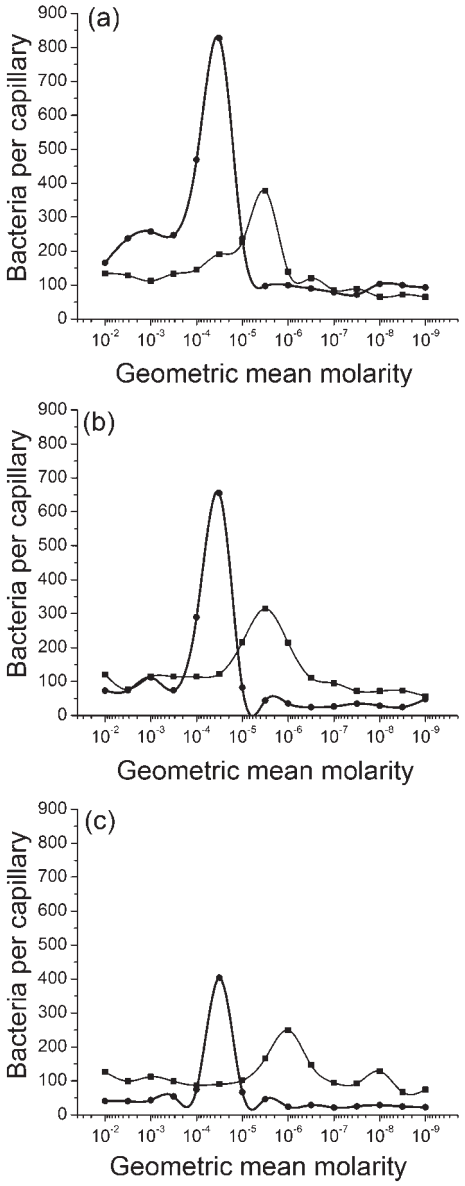

Fig. 2. Sensitivity assay results. (a) Comparison of the Gln371/Glu630/Glu637 (QEE) mutant (thin line, ■) and the Glu371/Glu630/Glu637 (EEE) strain (thicker line, ●). (b) Comparison of the Gln371/Gln630/Glu637 (QQE) mutant (thin line, ■) and the Glu371/Gln630/Glu637 (EQE) mutant (thicker line, ●). (c) Comparison of the Gln371/Glu630/Gln637 (QEQ) mutant (thin line, ■) and the Glu371/Glu630/Gln637 (EEQ) mutant (thicker line, ●).

Measuring kinase activity using the tethered cell assay

We also measured the counter-clockwise rotational bias of the flagellar motors using the tethered cell assay (Block *et al.*, 1983; Kirby *et al.*, 1999; Saulmon *et al.*, 2004). In this assay, the cells are tethered to a glass coverslip using an anti-flagellin antibody and their rotation is recorded using video microscopy at varying concentrations of asparagine. The average counter-clockwise rotational bias of the motors reflects the concentration of CheYp and thus provides an indirect measure of CheA kinase activity. It should be noted that these experiments were performed in the presence of asparagine, since in the absence of attractant, *cheRcheB* cells expressing only McpB rotate almost exclusively clockwise (Zimmer *et al.*, 2002).

When we looked for a general pattern among the data (Table 2), the results could best be classified based on the amidation state of site 371, the very site that affects the 'apparent K_d' of the receptor. When site 371 was encoded

Table 1. Apparent K_d values of *mcpB* mutants

K_d values were determined using the capillary sensitivity assay. In the experiments, a capillary containing asparagine is inserted into a suspension of bacteria containing 3.16-fold less asparagine. By counting the number of bacteria that accumulate in the capillary as a function of concentration, one can determine the K_d by determining the concentration at which accumulation is greatest. In the experiments involving the EQQ and QQQ mutants, no accumulation was observed at any concentration (denoted by ND). The raw data for the apparent K_d are shown in Fig. 2.

Receptor modification			K_d (μM)
371	630	637	
E	E	E	20
E	Q	E	20
E	E	Q	20
E	Q	Q	ND
Q	E	E	2
Q	Q	E	2
Q	E	Q	1
Q	Q	Q	ND

as a glutamate, the amidation of sites 630 and 637 decreased the bias at the two asparagine concentrations tested (25 and 130 μM). Moreover, the effect was additive; receptors with both sites 630 and 637 modified (371E/630Q/637Q) had a lower bias relative to the unmodified receptor (371E/630E/637E) than those with just one site modified (371E/630Q/637E and 371E/630E/637Q). When considering the two sites individually, however, we did not observe any significant difference between them. When site 371 was encoded as a glutamine, it was found that both sites 630 and 637 needed to be amidated in order to reduce the bias at the two concentrations tested (371Q/630E/637E versus 371Q/630Q/637Q). Individually, the modification state of these two sites did not lead to a significant reduction in the bias (371Q/630E/637E versus 371Q/630Q/637E and 371Q/630E/637Q). In summary, these results indicate that amidation of sites 630 and 637 reduces the ability of the receptor complex to stimulate the kinase.

Table 2. Rotational bias of McpB mutants

The rotational bias of different McpB mutants at two concentrations of asparagine was determined using the tethered cell assay. In these experiments, the cells were adhered to the glass coverslip by their flagella using an anti-flagellin antibody. The direction of cell rotation was recorded for approximately 5 min. Results are the mean ± SEM of at least 16 cells.

Receptor modification			Counter-clockwise bias (%)	
371	630	637	130 μM	25 μM
E	E	E	88.1 ± 0.7	60.1 ± 1.4
E	Q	E	71.6 ± 0.6	51.0 ± 1.2
E	E	Q	72.7 ± 0.9	48.1 ± 1.9
E	Q	Q	61.9 ± 1.2	41.4 ± 1.2
Q	E	E	80.9 ± 0.7	75.8 ± 1.4
Q	Q	E	79.8 ± 0.7	73.1 ± 1.0
Q	E	Q	77.7 ± 1.1	69.3 ± 1.1
Q	Q	Q	66.3 ± 0.9	66.1 ± 1.1

By contrast, amidation of site 371 mostly increased, rather than decreased, kinase activity (Table 2). In addition, we observed a much higher relative bias at 130 μM asparagine than at 25 μM in 371E strains as compared with 371Q strains. Presumably, most of the receptors are already titrated at 25 μM asparagine in the 371Q strains due to their lower 'apparent K_d'. The only exception to this rule is the case of 371Q/630E/637E, where the kinase is less active than the 371E/630E/637E case at 130 μM asparagine, but not at 25 μM. We cannot explain why amidation of site 371 decreases kinase activity at 130 μM asparagine, although we suspect that it is likely to be due to a confluence of competing effects. In particular, while the amidation of site 371 in general increases kinase activity it may also reduce electrostatic interactions as described below, leading in some cases to lower kinase activity.

Electrostatic surface potential of the receptor adaptation domain

Researchers at the Falke laboratory have previously proposed that electrostatic interactions along the dimer subunit interface of the adaptation region alter the stability and conformational dynamics of the *E. coli* aspartate receptor (Starrett & Falke, 2005; Swain *et al.*, 2009). According to their model, electrostatic repulsions between negatively charged glutamates repress the kinase by destabilizing helical packing within the adaptation region, having the effect of stabilizing the receptor where it interacts with the kinase and CheW. Likewise, neutralization of these negatively charged glutamates by amidation, and presumably methylation, activates the kinase by stabilizing the helical packing within the methylation region, having the effect of destabilizing the receptor where it interacts with the kinase and CheW.

Electrostatic interactions potentially explain some of the *in vivo* data regarding McpB. To explore this hypothesis in more detail, we compared the electrostatic surface potential of the solvent-exposed methylation region using homology models of McpB (Fig. 3). The examination of surface potential and isopotential patterns can reveal insights into the biochemical data (Honig & Nicholls, 1995). Similar to what has been found in both *E. coli* and *T. maritima* (Park *et al.*, 2006; Starrett & Falke, 2005), there is a distinct surface of negative potential that contains the adaptation sites in the cytoplasmic region of McpB. Comparison of the electrostatic surface potential for the adaptation region in different modification states indicates that the potential is primarily determined by sites 630 and 637. Specifically, the surface potential for the 371E/630E/637E cytoplasmic domain, which corresponds to the receptor that showed

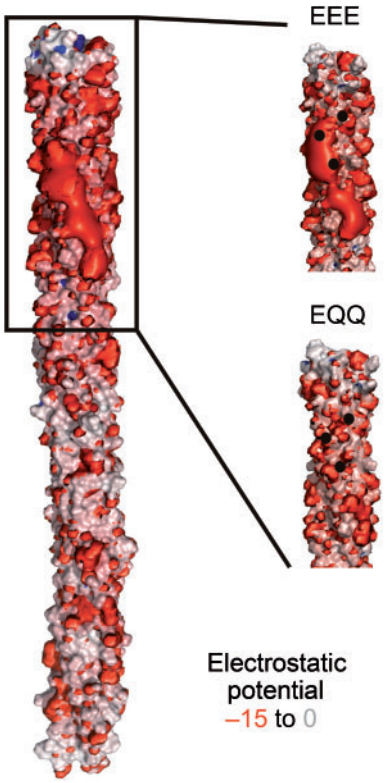

Fig. 3. Electrostatic potentials and isopotential contours of the solvent-accessible surface of McpB show a change in surface potential upon the neutralization of a negatively charged glutamic acid residue. The adaptation sites are shown as black circles. Clockwise from the top, site 371, site 630 and site 637, with the corresponding residue in that position labelled above.

the highest counter-clockwise bias in the tethered cell assay, shows a surface of negative potential that contains sites 630 and 637 but, interestingly, not site 371. When these two sites are changed to glutamines (371E/630Q/637Q), which corresponds to the receptor that showed the lowest bias in the tethered cell assay, the region of negative surface potential is greatly reduced. Based on the work of Falke and coworkers (Starrett & Falke, 2005), we hypothesize specifically that negatively charged glutamates at sites 630 and 637 destabilize the helical packing of the receptors. Amidation, and presumably methylation, of these two sites can then neutralize the negative charges and stabilize the helical packing of the receptors (Fig. 3).

In the case of site 371, we found that it had a much weaker effect on the surface potential than sites 630 and 637 (Fig. 3). This suggests that this site may affect receptor function by some other, still unknown mechanism.

DISCUSSION

Receptor methylation plays an integral role in bacterial chemotaxis. With the notable exception of *Helicobacter pylori* (Pittman *et al.*, 2001), all flagellated bacteria appear to employ some form of receptor methylation for chemotaxis (Alexander & Zhulin, 2007; Wuichet *et al.*, 2007), although the mechanistic details can vary significantly across species. Despite its prevalence, receptor methylation has only been extensively studied so far in *E. coli*. Little is known about this mechanism in other species of bacteria. In this work, we explored how covalent modification of the adaptation sites affects chemotaxis signalling in *B. subtilis*. The canonical asparagine receptor, McpB, has three adaptation sites, located at residues 371, 630 and 637 (Zimmer *et al.*, 2000). We found that the amidation of site 371 increased the sensitivity (i.e. binding affinity) of the receptor to asparagine whereas the corresponding changes to sites 630 and 637 had no substantive effect. We also found that the amidation of the negatively charged glutamates at sites 630 and 637 decreased kinase activity whereas the amidation of the glutamate at site 371, with a single exception, increased kinase activity (Table 2). Finally, the electrostatic surface potential of the cytoplasmic domain of McpB showed that negative charges at the adaptation sites might further explain the effect of covalent modifications on chemotactic ability.

The *B. subtilis* strains used in this study all contain CheD, which functions in the CheC/D/Y adaptation system by interacting with CheC when CheYp levels are high, effectively recruiting CheD away from the receptors. Past experiments have shown that mutants lacking *cheD* activate CheA kinase poorly (Kirby *et al.*, 2001). It is possible that the glutamate and glutamine substitutions at the three methylation sites may change the receptor's affinity for CheD, thereby influencing CheA kinase activity. Thus, the net kinase activity in the cell could reflect not only the direct change in the receptor caused by covalent modification of the adaptation sites but also the secondary effect of changing the receptor's affinity for CheD. However, preliminary experiments indicate that if there are such changes in affinity, they are slight.

As mentioned in Results, the reason that we were able to use chemotaxis assays to study receptor methylation is that there are two other adaptation systems in *B. subtilis*, the CheC/D/Y and CheV systems. These systems are redundant, with two sufficient for chemotaxis, albeit at reduced efficiency (Rao *et al.*, 2008). In our experiments, the methylation system was inactivated. Nonetheless, the cells were still able to migrate up gradients of attractant although with varying efficiencies based on the modification state (Fig. 2). Moreover, the cells were incapable of perfect adaptation (Table 2).

Role of electostatic interactions

As outlined in Results, the presence of negative charges (glutamates) at sites 630 and 637 greatly increases the surface negative potential of the adaptation region (Fig. 3). Based on a model proposed by the Falke laboratory for the

E. coli receptors, we hypothesized that these electrostatic interactions probably affect the intra- and inter-subunit packing of the *B. subtilis* receptors. However, the receptor–kinase complexes in *B. subtilis* and *E. coli* have reciprocal polarity. Attractant binding increases kinase activity in *B. subtilis* whereas it inhibits it in *E. coli*. This reciprocal polarity would potentially suggest that the *E. coli* model cannot be fully applied to *B. subtilis*. However, the differences in kinase activation most likely are due to how the two sets of receptors interact with the kinase and not to the receptors themselves. Evidence comes from dynamic receptor localization studies, where the binding of attractant has been shown to disrupt receptor packing in both *B. subtilis* and *E. coli* (Lamanna *et al.*, 2005). The packing was restored once the cells adapted to the attractant, presumably due in part to methylation and the concomitant neutralization of repulsive electrostatic interactions. Moreover, these results demonstrate that despite the differences in polarity, the same changes in receptor packing are observed in the two species of bacteria. Also, they demonstrate that these same changes lead alternatively to kinase activation in *B. subtilis* and kinase inhibition in *E. coli*, providing further evidence that the differences are due to how the receptors interact with the kinase and not the adaptation region, which directly affects packing. Finally, these patterns of reorganization are also consistent with our model where repulsive interactions between negatively charged glutamates at sites 630 and 637 destabilize the receptor and lead to increased kinase activity.

Dissimilarity between *B. subtilis* and *E. coli* is also intrinsic to the receptors themselves. In *E. coli*, substitutions of glutamines or glutamates at all methylatable positions both increase the kinase activity and the 'apparent K_d' of the receptor (Li & Weis, 2000; Sourjik & Berg, 2002a). In *B. subtilis*, however, we found that the modification state of sites 630 and 637 affects only the kinase activity but not the 'apparent K_d'. Only the amidation state of site 371 affects the 'apparent K_d'. Interestingly, we found in our structural modelling that the amidation state of site 371 does not affect the surface potential as greatly as amidation states of sites 630 and 637.

Implication of activation and sensitivity being decoupled from one another

As mentioned earlier, activity and sensitivity are not directly correlated with one another in McpB. In particular, there are modifications with high activity and high apparent affinity (371Q/630E/637E) and others with low activity and low apparent affinity (371E/630Q/637E and 371E/630E/637Q). Likewise, there are modifications with high activity and low apparent affinity (371E/630E/637E) and perhaps even one with low activity and high apparent affinity (371Q/630Q/637Q). In *E. coli*, on the other hand, there is a direct, inverse correlation between the two (Sourjik & Berg, 2002b, 2004). In the models commonly used to explain receptor activity in *E. coli*, the receptor complex is assumed to exist in one of two states: (1) a high-affinity, low-activity state and (2) a low-affinity, high-activity state (Keymer *et al.*, 2006; Mello & Tu, 2005; Rao *et al.*, 2004). The equilibrium partitioning between these two states is determined by the concentration of chemoattractant and degree of methylation/amidation. Our data for McpB, however, imply that the mechanism for receptor activation cannot be described by a simple two-state model but instead requires a more complicated model involving additional conformational states. While we still lack the requisite biochemical data to construct such a quantitative model, our results nonetheless suggest that *B. subtilis* is able to independently tune these two factors.

Receptor structure

How are activity and sensitivity decoupled from one another? In particular, why do modifications to site 371 affect the 'apparent K_d' whereas ones to site 630 and 637 do not? While the actual mechanism is still unknown, we note that the associated mechanism of attractant binding is different in *E. coli* and *B. subtilis*. In *E. coli*, attractants bind across the dimer interface and induce a piston-like movement in the descending helix, the one emerging from the transmembrane region (Chervitz & Falke, 1996; Yeh *et al.*, 1993). In *B. subtilis*, attractant binds within an individual monomer and induces a rotation between the helices (Glekas *et al.*, 2010; Szurmant *et al.*, 2004). Site 371 is located on the descending helix. Thus, modifications to it may affect the 'information flow' towards both the sensing domain and the kinase, located at the turn ('bottom') of the receptor (Fig. 4). By contrast, sites 630 and 637 are on the ascending helix. Modifications to these sites may affect just the information flow solely towards the kinase (Fig. 4). In the three-dimensional structural model, of course, the three sites appear to form a closely spaced triad. The close proximity of the sites suggests that they may be affected by electrostatic interactions between them. It also suggests that modification of each site may affect not only the conformation of each monomer of the cytoplasmic domain separately but also the interface of the two monomers that form the tightly wound dimer. Finally, comparing a sequence alignment of other *B. subtilis* receptors such as McpA and McpC, we find that the triad of putative methylation sites, and presumably mode of action, is conserved in other *B. subtilis* receptors (Le Moual & Koshland, 1996).

Comparison with previous work

Previously it was argued that the modification, amidation or methylation, of site 630 increased kinase activity whereas the modification of site 637 decreased it. These results were obtained from experiments where aspartate substitutions at each of the three sites and in combinations were examined using the tethered cell assay (Zimmer *et al.*, 2000). This approach was based on previous work from the Koshland lab (Shapiro & Koshland, 1994), in which glutamate/glutamine residues were substituted with aspartate residues

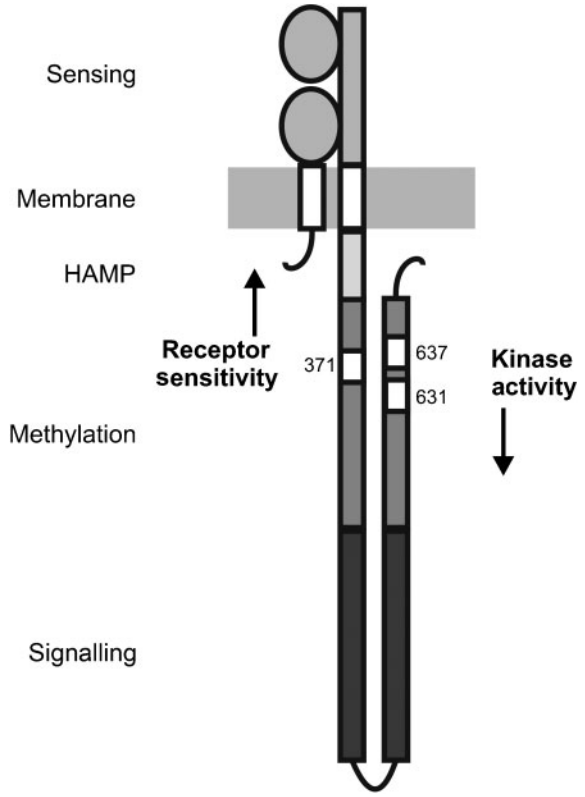

Fig. 4. Schematic representation of the McpB chemoreceptor monomer. The three adaptation sites are shown as white boxes. The cartoon shows that site 371 is in close proximity to the HAMP (histidine kinase, adenyl cyclase, methyl-accepting chemotaxis protein and phosphatase) domain (Aravind & Ponting, 1999), which can transmit signals to (and from) the sensing domain, the site where the ligand binds. Sites 630 and 637 can affect the signalling domain, which interacts with the kinase.

where a 'permanent' negative charge would be at the sites that could not be neutralized by methylation. Based on these previous studies, we anticipated that there would be some difference between sites 630 and 637 in the experiments reported in Tables 1 or 2, but evidently there is none. One possible explanation for this discrepancy is that previously employed aspartate-for-glutamate/glutamine substitutions, which are shorter by one methylene group, may alter the conformation of the receptor in some unnatural way. Considering the close proximity of the three adaptation sites, it does seem plausible that moving the negative charge from its position in a glutamate to its position in an aspartate (the distance of a methylene group or 1.33 Å) could have unnatural effects.

Model for site-specific methylation during taxis in a concentration gradient of attractant

Based on the results of this work, we propose the following model for site-specific methylation. In the absence of attractant, we expect that site 371 is either amidated (i.e. glutamine) or methylated and sites 630 and 637 are unmethylated (i.e. glutamates). Such a modification state would be optimal in the sense that the kinase is maximally active and the 'apparent K_d' lowest. Thus, the bacteria would be able to detect a concentration gradient of attractant beginning at quite low concentrations. As the bacterium swam up the gradient, site 371 would gradually be deamidated when it is a glutamine or demethylated when a methyl-glutamate. This would have the effect of reducing kinase activity due to higher ambient concentrations of asparagine as part of the adaptation process and also increasing the 'apparent K_d', enabling the bacterium to optimally sense gradients at higher concentrations of attractant. Similarly, we expect that sites 630 and 637 would gradually become more methylated (for which amidation was used in this study as a mimic). This would have the effect of further reducing kinase activity as part of the adaptation process. Based on the relative timing of the demethylation and methylation steps (Kirby *et al.*, 1999), we expect that changes at site 371 would occur more rapidly than those at sites 630 and 637. The reason why these two processes occur on different timescales, however, is still unknown.

Conclusions

In summary, we have found that amidation of site 371 increases McpB's apparent affinity for asparagine and also, in most cases, increases kinase activity. In addition, we found that amidation of sites 630 and 637 decreases kinase activity but does not affect the apparent affinity. These findings further our understanding of the site-specific methylation system in *B. subtilis* by demonstrating how the modification of specific sites can have varying effects on receptor function.

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