Selective Methylation Changes on the *Bacillus subtilis* Chemotaxis Receptor McpB Promote Adaptation*

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The *Bacillus subtilis* McpB is a class III chemotaxis receptor, from which methanol is released in response to all stimuli. McpB has four putative methylation sites based upon the *Escherichia coli* consensus sequence. To explore the nature of methanol release from a class III receptor, all combinations of putative methylation sites Gln<sup>371</sup>, Gln<sup>509</sup>, Glu<sup>639</sup>, and Glu<sup>637</sup> were substituted with aspartate, a conservative substitution that effectively eliminates methylation. McpB<sub>(E630D,E637D)</sub> in a ∆(mcpA mcpB tlpA tlpB)101::cat mcpC4::erm background failed to release methanol in response to either the addition or removal of the McpB-mediated attractant asparagine. In the same background, McpB<sub>(E630D,E637D)</sub> produced methanol only upon asparagine addition, whereas McpB<sub>(Q371D,E630D) E637D</sub> produced methanol only upon asparagine removal. Thus methanol release from McpB was selective. Mutants unable to methylate site 637 but able to methylate site 630 had high prestimulus biases and were incapable of adapting to asparagine addition. Mutants unable to methylate site 630 but able to methylate site 637 had low prestimulus biases and were impaired in adaptation to asparagine removal. We propose that selective methylation of these two sites represents a method of adaptation novel from *E. coli* and present a model in which a charged coiled coil containing the methylation and signaling regions (Fig. 1). We propose that CheC, a protein not found in enteric systems, has a role in regulating this selective methylation.

Chemotaxis is the process by which motile bacteria sense their environment and move toward more favorable surroundings. This is accomplished by altering the direction of flagellar rotation in response to changes in effector gradients. Information about the outside of the cell is transduced to the inside via transmembrane receptors called methyl-accepting chemotaxis proteins. Both ligand binding to the extracellular portion of the receptor and methyl modification of the intracellular portion induce conformational changes that modulate the activity of an associated histidine kinase, CheA (1). CheA activity in turn regulates phosphorylation of the response regulator CheY, the activated form of which binds to the motor switch to promote smooth swimming (1, 2). However, bacteria respond only to changes in their environment. In the presence of unchanging stimuli, the transduced signal is shut off despite the fact that ligand is still bound. This ability to adapt is essential for chemotaxis and allows cells to respond to ever changing environmental conditions.

In *Escherichia coli*, adaptation depends largely upon the reversible methyl esterification of certain glutamate residues that serve to counteract the effects of bound ligand (3–5). The methyltransferase CheB, which is also CheA-activated, demethylates the receptor to promote adaptation to attractant removal. Receptor demethylation results in the release of volatile methanol from the cell (6–9). CheB also functions as a deamidase that converts the nascent glutamine residues of some sites to glutamates, which in turn serve as sites for CheR-mediated methylation (3). A dedicated methyltransferase, CheR, methylates the receptor using S-adenosylmethionine as the methyl donor to promote adaptation to attractant addition (10, 11). A deletion in either of these genes results in cells that cannot adapt to stimuli (12).

Mechanistically, the binding of attractant to the N-terminal sensing domain of the *E. coli* aspartate receptor Tar is believed to cause a 1.6 Å downward displacement with a 5° tilt in the second transmembrane spanning helix (TM2) of one monomer relative to the other transmembrane helices of the homodimeric receptor complex. This piston-like displacement may be propagated down the length of the receptor because of its predominantly α-helical nature and thus affect CheA activity (13–16). The cytosolic C terminus is an antiparallel coiled-coil containing the methylation and signaling regions (Fig. 1). Methylation of the receptor presumably causes a compensatory shift in TM2 that restores basal CheA activity.

Reversible receptor methyl esterification is also implicated in adaptation in *Bacillus subtilis*. However, deletions of cheB and cheR merely impair adaptation, particularly at high receptor occupancy (12). This has led to speculation that *B. subtilis* has both methylation-dependent and methylation-independent adaptation systems (9). Unlike *E. coli*, methanol is released both upon the addition and the removal of all attractants tested, a result that suggests the mechanism of adaptation is different between the two organisms. Moreover, adaptation also involves CheC, a protein with no *E. coli* counterpart (18–20).

Sequence alignments reveal three distinct classes of receptor (21). Class III receptors are typified by the presence of four 14-amino acid insertion/deletion (INDEL) regions located within the methylation and signaling regions. Class II receptors have only INDELS 2 and 3 within the signaling region,

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1 The abbreviations used are: INDEL, insertion/deletion; CB, chemotaxis buffer; kb, kilobase.
whereas class I receptors contain no INDELs (Fig. 1). These INDEL regions are postulated to give the antiparallel coiled-coil receptor helices extra length without altering their orientation (21). Enteric bacteria such as E. coli and Salmonella typhimurium have class I receptors that release methanol in response to negative stimuli while suppressing methanol release in response to positive stimuli (22, 23). The class III receptors found in the Gram-positive B. subtilis and in the archaeon Halobacterium salinarum release methanol in response to all stimuli (24–27). Moreover, the B. subtilis receptor McpB undergoes net demethylation followed by net remethylation in response to the addition of asparagine such that the level of receptor methylation in the prestimulus and adapted states is the same (20). For class I receptors, attractants cause a net increase in receptor methylation, whereas repellents cause a net decrease (28, 29).

The restoration of receptor methylation in B. subtilis following the addition of asparagine suggested that selective methylation changes might be taking place at a specific subset of sites on the unbound versus bound receptor. To explore this possibility, all combinations of glutamines/glutamates within the four putative consensus sites reported by Hanlon and Ordal (30) were substituted with asparagine. Similar substitutions in the E. coli receptor Tar were very poorly methylated (31). In this way, we ensure a fixed negative charge at a given site. If the selective methylation hypothesis is true, then some mutants should greatly affect methanol release and adaptation to attractant addition, whereas other mutants should affect methanol release and adaptation to repellent removal.

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains and Plasmids**—All bacterial strains and plasmids used in this study are summarized in Tables I and II. All plasmids were propagated in E. coli strain TG-1 (Amersham Pharmacia Biotech). Chemicals, Solutions, and Growth Media—L-[3H]Methionine (80–85 Ci mmol⁻¹) was purchased from Amersham Pharmacia Biotech. All other chemicals were reagent grade. Luria-Bertani (LB) medium is 1% tryptone, 0.5% yeast extract, and 1% NaCl. Chemotaxis buffer (CB) is 0.1 mM EDTA, 50 μM CaCl₂, 0.05% glycerol, 5 mM sodium lactate, 0.3 mM ammonium sulfate, and 20 mM potassium phosphate, pH 7.0 (32). Protoplast buffer is 20% sucrose, 25 mM potassium phosphate, 10 mM MgCl₂, 30 mM sodium lactate, and 1.0 mM EDTA, pH 7.0 (33). Minimal medium is 50 mM potassium phosphate, 1 mM (NH₄)₂SO₄, 1.2 mM MgCl₂, 140 μM CaCl₂, 10 μM MnCl₂, 50 μg/ml required amino acids, and 20 mM sorbitol, pH 7.0 (32).

**Construction of Aspartate Substitution Plasmids**—A long polymerase chain reaction was performed on pAIN700 to create the various aspartate substitution mutants using the following five-phosphorylated primers (Integrated DNA Technologies) to generate pING1 through pING1234, where the number denotes an aspartate substitution at the indicated site (numbered 1 through 4 from the N to the C terminus) (34): Q371D-F, 5'-GATCTTACCCGATCTGCGCCGGCAGACGAGTA-3'; Q371D-R, 5'-TTCCGGAAGATGCCGGCACATTGTCACCG-3'; Q585D-F, 5'-ATCTGTCAAGACCTTCAACGATGTTTACG-3'; Q585D-R, 5'-CTTCGACGCGTCTATTCGCTGACGTTGCCG-3'; E630D-F, 5'-CATGGAAGAATGCGACCGAGACGAGTA-3'; E630D-R, 5'-CTAGCGACGAGACGAGACGAGACGAGTA-3'; E637D-F, 5'-TACGCGACGAGACGAGACGAGACGAGTA-3'; E637D-R, 5'-CATGGAAGAATGCGACCGAGACGAGTA-3'.

The pAIN700 was subjected to polymerase chain reaction using the above primers, which were designed to create a new restriction site at the ligation junction to make the mutant identifiable by restriction digest. The polymerase chain reaction product was then ligated, digested with Dpn1 to remove template plasmid, and then transformed into TGI E. coli. Amp⁴ candidate colonies were screened using the introduced restriction sites (BglII for Q371D and Q585D, NheI for E630D, and EcoRV for E637D). The pertinent region of each single substitution mutant was sequenced at the W. M. Keck Center for Comparative and Functional Genomics. Double, triple, and quadruple substitutions were created in an iterative process. The proximity of site Glu⁴⁰⁰ to Glu⁴³⁷ required the use of a special forward primer to create the E630D/E637D double mutant. 2.8-kb EcoRI/NotI fragments containing the mutant mcpB alleles were subcloned into pAIN750 digested with the same enzymes to generate pONG1 through pONG1234. pAIN750 is a modified version of the amyE integration vector pDING750 (17).

**Construction of Asp-expressing Strains**—The various mcpB alleles were crossed into the amyE locus of OI3180 (∆mcpB mcPB tlpB) by linearizing the respective pONG subclones with AarII, transforming into OI3180, and selecting for Cm²⁸ Spec⁺ Erm⁺ Amy⁻ (35). The above strains were then crossed with OI3280 chromosomal DNA, selecting for Erm⁺ colonies to place all mutant mcpB alleles in a ∆mcpB mcPB tlpB101::cat mcPC4::erm background.

**Pulse-labeled Methylation**—Experiments were performed as described (33). Briefly, 100 μl of a 2-ml overnight culture was added to 10 ml of LB, incubated at 37 °C, and grown to early stationary phase. Cells were washed twice in CB with 250 μg ml⁻¹ chloramphenicol, once in protoplast buffer with 250 μg ml⁻¹ chloramphenicol, and resuspended...
to $A_{600} = 1.0$ in 3 ml of protoplast buffer with 250 $\mu$g ml$^{-1}$ chloramphenicol and 4 mg ml$^{-1}$ lysozyme. Cells were shaken at 250 rpm at 37°C for 20 min following the addition of 150 $\mu$l of 1 $\mu$M L-$\text{[3H]methionine}$. 1-ml aliquots were removed and flash-frozen in dry ice/acetone baths. Samples were then thawed, and centrifuged at 3000 x g for 45 min at 4°C. Pelleted membranes were resuspended in 100 $\mu$l of 4 x SDS solubilizer, boiled for 7 min, and electrophoresed at 15 V cm$^{-1}$ through 10% SDS-polyacrylamide gels, pH 8.8 (36). Gels were treated for fluorography as described (37).

Western blot analysis of McpB—Cells were treated as outlined for pulse label methylations except that no L-$\text{[3H]methionine}$ was added. Western analysis of mcPB mutants was performed as described (38). Antigen-antibody was detected using Supersignal (Pierce). Visualiization required a 5-s to 1-min exposure of Kodak X-Omat AR film.

Continuous flow assay—The continuous flow assay has been described (39). Briefly, 100 $\mu$l of a 2-ml overnight culture was added to 10
Table III

| Mutant            | Delay (min) |
|-------------------|-------------|
| McpB              | 0           |
| McpB<sub>(E630D)</sub> | 0.3         |
| McpB<sub>(E637D)</sub> | 0.9         |
| McpB<sub>(E630D,E637D)</sub> | 0.3         |
| McpB<sub>(Q371D,E630D)</sub> | 0.9         |
| McpB<sub>(Q371D,E637D)</sub> | 0.6         |

ml of LB, incubated at 37 °C, and grown to early stationary phase. Cells were washed three times in CB with 250 μg ml<sup>-1</sup> chloramphenicol and resuspended to an<sub>opt</sub> = 1.0 in 3 ml of CB with 250 μg ml<sup>-1</sup> chloramphenicol. Cells were shaken at 250 rpm at 37 °C for 20 min following the addition of 150 μl of 1 μM 1-[methyl-<sup>3</sup>H]methionine. Radiolabeled cells were then transferred onto a 0.45-μm Nalgene filter and connected to the continuous flow apparatus at a flow rate of 15 ml min<sup>-1</sup>. Cells were pretreated with 504 μM asparagine in CB with 250 μg ml<sup>-1</sup> chloramphenicol for 4 min and then switched back to CB with chloramphenicol only for 6 min before the assay was initiated by collecting 0.3-min fractions. 504 μM chloramphenicol was added at t = 5 and t = 15 min and removed at t = 10 and t = 20 min. A 0.4-ml aliquot of each fraction was transferred to lidless 0.5-ml Eppendorf tubes, which were in turn placed into scintillation vials containing 2.5 ml of scintillation fluid. Vials were sealed and volatile methanol was allowed to equilibrate with the scintillation fluid for 36 h before counting. Chemotaxis to asparagine is solely mediated through McpB. The value of 504 μM asparagine is predicted to titrate 90% of the McpB receptors based upon the experimentally determined K<sub>v</sub> value (40).

**Tethered Cell Assay**—The tethered cell assay has been described (20). Strains were grown on tryptose blood agar base plates ON in a 30 °C wet incubator. Cells were taken straight off the plate and suspended in minimal medium. The suspension was then diluted to an<sub>opt</sub> = 0.014 in 25 ml of minimal medium and grown at 37 °C with vigorous shaking (250 rpm) for 4.5 h. 15 min prior to harvesting, 200 μl of a 5% glycerol, 0.5 μl sodium lactate solution was added to the culture. The cells were blended at full speed in a Waring blender for 10 s to shear the flagella. A 250-μl aliquot of blended cells was placed atop a glass coverslip pretreated with 504 μg ml<sup>-1</sup> chloramphenicol for 4 min and then switched back to CB with chloramphenicol only for 6 min before the assay was initiated by collecting 0.3-min fractions. 504 μM asparagine was added at t = 5 and t = 15 min and removed at t = 10 and t = 20 min. A 0.4-ml aliquot of each fraction was transferred to lidless 0.5-ml Eppendorf tubes, which were in turn placed into scintillation vials containing 2.5 ml of scintillation fluid. Vials were sealed and volatile methanol was allowed to equilibrate with the scintillation fluid for 36 h before counting. Chemotaxis to asparagine is solely mediated through McpB. The value of 504 μM asparagine is predicted to titrate 90% of the McpB receptors based upon the experimentally determined K<sub>v</sub> value (40).

**RESULTS**

**Effect of Triple and Quadruple Aspartate Substitutions on Methanol Release**—To test whether an aspartate substitution at a given site prevents its methylation, the quadruple aspar- tate mutant was tested in the continuous flow assay. In this experiment, the evolution of [<sup>3</sup>H]methanol was followed by collecting eluant fractions as buffer or buffer plus asparagine was passed over cells caught on a 0.45-μm filter (see “Experimental Procedures”). As expected, McpB<sub>(Q371D,E630D,E637D)</sub> had no [<sup>3</sup>H]methanol release upon either the addition or removal of asparagine (data not shown). McpB<sub>(Q371D,E630D)</sub> also failed to release [<sup>3</sup>H]methanol (Fig. 2). Therefore, site 595 did not contribute to McpB methanol production and is not likely a site of methylation. Given the deviation of this site from the methylation consensus sequence, this was not too surprising (Fig. 1). Moreover, no methylation was observed for McpB<sub>(Q371D,E630D,E637D)</sub> in pulse label methylation experiments (data not shown; see “Experimental Procedures”). Thus [<sup>3</sup>H]methanol production from McpB was attributable only to sites 371, 630, and 637. Western blot analysis using anti-McpB antibody confirmed approximate wild-type expression for all mcP B mutants used in this study (data not shown). All experiments were done in a ΔmcpA McpB<sub>(Q595D)</sub> background. Experiments were performed as described under “Experimental Procedures.” A, methanol production from wild-type McpB. B, methanol production from McpB<sub>(Q371D,E630D,E637D)</sub>. Downward arrows indicate the addition of 504 μM asparagine (90% receptor occupancy). Upward arrows represent asparagine removal.

**Effect of Single Aspartate Substitutions on Methanol Re-lease**—To determine the contribution of individual sites on methanol production, continuous flow assays were performed on each of the single substitution mutants. All mutants released [<sup>3</sup>H]methanol upon both the addition and removal of asparagine (Fig. 3). McpB<sub>(Q595D)</sub> had wild-type [<sup>3</sup>H]methanol production, a result consistent with it not being a site of methylation (Fig. 3B). However, the release of [<sup>3</sup>H]methanol was markedly diminished for the remaining mutants. We conclude that no site is solely responsible for [<sup>3</sup>H]methanol release upon either the addition or removal of asparagine.

**Effect of Double Aspartate Substitutions on Methanol Re-lease**—Greater insight into the B. subtilis methylation system has come from the double aspartate substitution mutants. McpB<sub>(Q595D)</sub> failed to release [<sup>3</sup>H]methanol upon asparagine addition, whereas leaving [<sup>3</sup>H]methanol release upon asparagine removal intact (Fig. 4A). Thus sites 371 and 630 were responsible for [<sup>3</sup>H]methanol release upon asparagine addition,
whereas the unaltered site 637 only contributed to [3H]methanol release upon asparagine removal. However, McpB (Q371D,E637D) released [3H]methanol upon both asparagine addition and removal. Unaltered site 630 was therefore indiscriminate and contributed to [3H]methanol release in both cases (Fig. 4B). Finally, McpB(E630D,E637D) released [3H]methanol upon asparagine addition but failed to release [3H]methanol upon asparagine removal (Fig. 4C). Thus sites 630 and 637 were responsible for [3H]methanol release upon asparagine removal, whereas the unaltered site 371 only contributed to [3H]methanol release upon asparagine addition.

It has been demonstrated for the E. coli receptor Tar that aspartate substitutions have polar effects on nearby sites. Specifically, methylation was nearly eliminated at sites seven residues N-terminal to a mutated site (31). We do not know the
behind that of the native receptor. [3H]Methanol release for an alcohol release upon asparagine removal for some mutants lagged McpB(Q371D,E637D) was delayed by about 30 s. McpB(Q371D) and a minute relative to wild-type. The removal response of mutant in vivo esterase functionally complements its respective E. coli receptors are known to migrate at class I and class III receptors. Such a difference is particularly interesting because B. subtilis CheR and CheB methylate and demethylate E. coli receptors in vitro (11, 42), and the methyl-esterase functionally complements its respective E. coli null mutant in vivo (9). E. coli receptors are known to migrate at different rates through SDS-polyacrylamide gel electrophoresis depending on their degree of methylation (43). McpB, however, runs as a single band that splits into a doublet upon asparagine addition and returns to a single band following asparagine removal (20). This suggested that McpB might predominantly have two methylated states and that the receptor is methylated selectively upon asparagine addition and removal. If this hypothesis is true, then methanol release upon the addition and removal of asparagine (90% receptor occupancy), and upward arrows indicate asparagine removal.

Delayed Methanol Release upon Asparagine Removal—Methanol release upon asparagine removal for some mutants lagged behind that of the native receptor. [3H]Methanol release for both McpB(E630D) and McpB(Q371D,E630D), were delayed by about a minute relative to wild-type. The removal response of McpB(Q371D,E637D) was delayed by about 30 s. McpB(Q371D) and McpB(E637D) both had prestimulus biases of ~70% and both failed to adapt to asparagine addition. De-excitation and adaptation to asparagine removal was largely unaffected. However, McpB(Q371D,E630D) had a more pronounced removal response (Fig. 5A). We conclude that methylation of site 637 is involved in adaptation to the addition of asparagine.

Behavioral Effects of the McpB(E630D) and McpB(Q371D,E630D) Mutants—To explore the behavioral consequences of a fixed negative charge at the various sites, we tested the asparagine substitution mutants in the tethered cell assay. In this assay, cells are tethered to a coverslip us striping anti-flagella antibody. The coverslip is then placed over a laminar flow chamber, and rotational data are collected as buffer or buffer plus asparagine is passed over the cells (see "Experimental Procedures"). McpB(E630D) and McpB(Q371D,E637D) both had prestimulus biases of ~70% and both failed to adapt to asparagine addition. McpB(E630D,E637D) and McpB(Q371D,E630D,E637D) were particularly striking with a delay of ~2 and 1.6 min, respectively. McpB(E630D) appeared almost wild-type (Fig. 5C). We note that the effect of a fixed negative charge at site 637 (high bias and failure to adapt to asparagine addition) is only manifest when site 637 can be methylated. Conversely, the effect of a fixed negative charge at site 630 (low bias and failure to adapt to asparagine removal) is only manifest when site 637 can be methylated.

**DISCUSSION**

The observation that B. subtilis releases methanol in response to all stimuli is significant because it suggests that the role of receptor methylation is different from that of enteric organisms. This may underscore a general difference between class I and class III receptors. Such a difference is particularly interesting because B. subtilis CheR and CheB methylate and demethylate E. coli receptors in vitro (11, 42), and the methyl-esterase functionally complements its respective E. coli null mutant in vivo (9). E. coli receptors are known to migrate at...
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TABLE IV
Prestimulus bias of McpB aspartate mutants

| Mutant | 371 | 630 | 637 | % clockwise bias |
|--------|-----|-----|-----|-----------------|
| McpB<sub>Q371D</sub> | D | D | E | 39 |
| McpB<sub>E630D</sub> | Q | D | E | 40 |
| McpB<sub>E630D,E637D</sub> | D | D | D | 46 |
| McpB<sub>Q371D</sub> | E | E | E | 50 |
| McpB<sub>E630D,E637D</sub> | Q | D | D | 55 |
| McpB<sub>Q371D</sub> | Q | E | E | 57 |
| McpB<sub>E637D</sub> | Q | E | D | 67 |
| McpB<sub>Q371D,E637D</sub> | D | E | D | 72 |

TABLE V
Delayed tumble response of McpB aspartate mutants

| Mutant | Delay (min) |
|--------|-------------|
| McpB   | 0           |
| McpB<sub>Q371D</sub> | 0.7 |
| McpB<sub>E630D</sub> | 1.9 |
| McpB<sub>E637D</sub> | 1.2 |
| McpB<sub>Q371D,E630D</sub> | 0.7 |
| McpB<sub>Q371D,E637D</sub> | 1.2 |
| McpB<sub>E630D,E637D</sub> | 2.0 |
| McpB<sub>Q371D,E630D,E637D</sub> | 1.6 |

and 630 upon asparagine addition and from sites 630 and 637 upon asparagine removal.

Methanol release, as measured in the flow assay, does not represent a single demethylation event but rather an increased level of methyl turnover at susceptible sites (38). No site was entirely responsible for methanol release upon either asparagine addition or removal (Fig. 3). However, the double substitution mutants reveal that site 371 was subject to demethylation only upon asparagine addition, whereas site 637 was subject to demethylation only upon asparagine removal. Site 630 appeared indiscriminate and was demethylated in both cases. It is important to point out that being subject to methyl turnover does not necessarily mean that a site was methylated to begin with. These results imply that, in the transition period between the addition of asparagine and the return to basal methanol production, the susceptible sites are 371 and 630. In the transition period between the removal of asparagine and the return to basal methanol production, the susceptible sites are 630 and 637. This suggests that the receptor conformational change following attractant addition to the adapted state is different from the conformational change following attractant removal.

Class III receptors are thought to closely resemble their class I counterparts with only the addition of four 14-amino acid INDEL regions, which serve to extend the length of the anti-parallel coiled-coil structure without affecting the relative orientations of the helices (21). This places site 630 and site 637 11 Å apart on the same face of the coiled-coil helix (Fig. 1). The fact that these two sites were selectively demethylated is most striking and strongly supports a model involving selective receptor methylation in B. subtilis.

All McpB mutants exhibiting a methylation phenotype were tested in the tethered cell assay to determine the in vivo consequences of forced negative charges at the various sites. It was most interesting to see that site 630 and site 637 had an “opposite” global effect on chemotaxis. Mutants in site 637 (when site 630 was unchanged) had higher than normal (~70%) prestimulus biases and failed to adapt to the addition of asparagine. Mutants in site 630 (when site 637 was unchanged) had low (~40%) biases, and the McpB<sub>E630D</sub>, mutant in particular had a marked inability to adapt to asparagine removal. These phenotypes were only manifest when the adjacent site was unchanged. Mutants having both or neither sites 630 and 637 altered had more nearly wild-type biases and were capable of adaptation. Because a methylated glutamate residue is neutral, adaptation appears to involve switching the negative charge between these two sites.

These observations lead to the following electrostatic model for B. subtilis adaptation (Fig. 6). We propose that there is a fixed charged residue on one monomer of the functional homodimer that is strategically placed between sites 630 and 637 of the other monomer. This charge can be either positive or negative. Here the model is presented with a fixed negative charge promoting selective electrostatic repulsions. However, an analogous construction can be made using a fixed positive charge that promotes selective electrostatic attractions. We believe that attractant binding results in a conformational change in McpB that shifts one monomer up relative to the other to activate CheA (as opposed to E. coli, where the addition of attractant results in the TM2 region of one monomer shifting down to inhibit CheA) (13–16). This results in demethylation of site 630. Adaptation to the addition of attractant is achieved by methylating site 637 but not site 630. This would cause a compensatory conformational change that balances the effect of bound ligand. In the model, we visualize this as a shifting of the monomer back down because of the elimination of electrostatic repulsion between the glutamate at site 637 and the hypothesized negative charge. Removal of attractant would cause the monomer to shift down relative to the other to inhibit CheA. This results in demethylation of site 637. Adaptation to attractant removal is achieved by methylating site 630 but not site 637. This causes a compensatory conformational change back to the original state. In the model, we visualize this as a restoring upward shift of the monomer because of the elimination of electrostatic repulsion between the glutamate at site 630 and the putative negative charge.

Single aspartate substitutions at sites 630 or 637 have opposing phenotypes because of opposing electrostatic effects from the hypothesized charge. Aspartate substitution at both sites would fail to generate a net force in either direction and would thus have a phenotype somewhere in between. The model is thus consistent with earlier findings in B. subtilis that show that cheB and cheR mutants are capable of adaptation by a methylation-independent mechanism (9). Because sites 630 and 637 are glutamates, cheB and cheR mutants would result in both of these sites being either methylated or unmethylated, respectively. The system is apparently designed to be robust, and the loss of either enzyme results in a receptor that defaults into a conformation that can at least partially adapt through the methylation-independent mechanism. The methylation-independent mechanism appears sufficient for adaptation to low concentrations of asparagine, as taxis in a cheB mutant in the capillary assay is nearly normal at low concentrations (38).

Because B. subtilis and E. coli CheB and CheR appear to be interchangeable, it stands to reason that something is regulating selective methylation at a level above these enzymes. Obvious candidates for this are CheC and CheD, two proteins found in B. subtilis and the archaea, but not found in E. coli. Both CheC and CheD appear to bind the cytosolic portion of McpB<sup>2</sup> and are known to affect receptor methylation (18). Interestingly, a cheC mutant has a phenotype that is almost identical to that of the aspartate substitution at site 637 in that both have markedly high prestimulus biases and fail to adapt to the addition of attractant (Fig. 5A, 18). It is therefore possible that CheC is required to facilitate methylation of this site.

Methylation sites 371 and 637 are immediately adjacent to

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<sup>2</sup> C. J. Kristich and G. W. Ordal, unpublished data.
INDEL regions 1 and 4 (Fig. 1), and there is a correlation between the presence of these INDELs and the presence of both CheC and CheD. This suggests two possible mechanisms for regulating selective methylation. The first involves exposing sites because of induced changes in receptor conformation. We imagine that the selective binding of CheC or CheD to these INDELs could determine whether a particular site is subject to methylation by causing it to be either buried at the interface between the two monomers (thus protected from modification) or exposed because of a twisting motion. Another mechanism might involve steric interference. The binding of CheC or CheD to the INDELs could cause them to overlap the consensus sequences of sites 371 and 637 and thus deny the methylesterase and/or the methyltransferase access to the sites. Such a binding event could be dependent upon the methylated state of the receptor.

Lastly, it is tempting to speculate on the source of the putative charge. Conceivably, it could come from a residue on one monomer of the homodimer, from a protein that binds to one monomer and thus fixes a negative charge between sites 630 and 637 of the other, or even be the result of a post-translational modification. Kim et al. (44) has recently crystallized the ...
C terminus of an *E. coli* serine receptor Tsr in which all sites of methylation were changed to glutamine residues. They report possible hydrogen bonding contacts between the glutamines representing methylation sites Gln\(^{297}\), Glu\(^{304}\), and Gln\(^{311}\) on the first methylated helix of one monomer of the homodimer with residues Glu\(^{479}\), Gln\(^{472}\), and Glu\(^{465}\) on the second methylated helix of the other monomer (44). Assuming these hydrogen bonding contacts lie on the dimeric interface, the multiple sequence alignment of Le Moual and Koshland (21) would implicate the lysine residue at position 381. It is also possible that the putative charge comes from an auxiliary protein bound to one monomer of the homodimer. The proximity of INDEL regions 1 and 4 to the sites of methylation make CheC and CheD attractive candidates.

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REFERENCES
1. Garrity, L. F., and Ordal, G. W. (1997) *Microbiology* 143, 2945–2951
2. Bischoff, D. S., Bourret, R. B., Kirsch, M. L., and Ordal, G. W. (1985) *Biochemistry* 24, 9256–9261
3. Terwilliger, T. C., and Koshland, D. E., Jr. (1984) *J. Biol. Chem.* 259, 7719–7725
4. Terwilliger, T. C., Bugones, E., Wang, E. A., and Koshland, D. E., Jr. (1983) *J. Biol. Chem.* 258, 9608–9611
5. Ahlgren, L. A., and Ordal, G. W. (1983) *Biochem. J.* 213, 759–763
6. Stock, J. B., and Koshland, D. E., Jr. (1978) *Proc. Natl. Acad. Sci. U. S. A.* 75, 3659–3663
7. Fuhrer, K. F., and Ordal, G. W. (1991) *J. Bacteriol.* 173, 7443–7448
8. Goldman, D. J., Nettleton, D. O., and Ordal, G. W. (1984) *Biochemistry* 23, 675–680
9. Kirsch, M. L., Peters, P. D., Hanlon, D. W., Kirby, J. R., and Ordal, G. W. (1993) *J. Biol. Chem.* 268, 18610–18916
10. Springer, W. R., and Koshland, D. E., Jr. (1977) *Proc. Natl. Acad. Sci. U. S. A.* 74, 533–537
11. Burgess-Cassler, A.,ullah, A. H. J., and Ordal, G. W. (1982) *J. Biol. Chem.* 257, 8412–8417
12. Kirsch, M. L., Zuberi, A. R., Henner, K., Peters, P. D., Yadzi, M. A., and Ordal, G. W. (1993) *J. Biol. Chem.* 268, 25350–25356
13. Lynch, B. A., and Koshland D. E., Jr. (1991) *Proc. Natl. Acad. Sci. U. S. A.* 88, 18409–18416
14. Falke, J. J., Dernberg, A. F., Sternberg, D. A., Zalkin, N., Milligan, D. L., and Koshland, D. E., Jr. (1988) *J. Biol. Chem.* 263, 14850–14858

15. Danielson, M. A., Biemann, H.-P., Koshland, D. E., Jr., and Falke, J. J. (1994) *Biochemistry* 33, 6100–6109
16. Chait, S., and Falke, J. J. (1996) *Proc. Natl. Acad. Sci. U. S. A.* 93, 2545–2550
17. Guerout-Fleury, A.-M., Frandsen, N., and Strager, P. (1996) *Gene (Amst.)* 180, 57–61
18. Rillaro, M. M. L., Kirby, J. R., Bochar, D. A., and Ordal, G. W. (1995) *Biochemistry* 34, 3823–3831
19. Rosario, M. M. L., and Ordal, G. W. (1996) *Mol. Microbiol.* 21, 511–518
20. Kirby, J. R., Saulmon, M. M., Kristich, C. J., and Ordal, G. W. (1999) *J. Biol. Chem.* 274, 11092–11100
21. Le Moual, H., and Koshland, D. E., Jr. (1996) *J. Mol. Biol.* 261, 568–585
22. Toews, M. L., Guy, M. F., Springer, M. S., and Adler, J. (1979) *Proc. Natl. Acad. Sci. U. S. A.* 81, 5544–5548
23. Kehry, M. R., Doak, T. G., and Dahquist, F. W. (1984) *J. Biol. Chem.* 259, 11828–11835
24. Wong, L. S., Johnson, M. S., Sandberg, L. B., and Taylor, B. L. (1995) *J. Bacteriol.* 177, 4342–4349
25. Kirby, J. R., Kristich, C. J., Feinberg, S. L., and Ordal, G. W. (1997) *Mol. Microbiol.* 24, 869–878
26. Nordmann, B., Lebert, M. R., Alam, M., Nitz, S., Kollmannsberger, H., Oesterhelt, D., and Hazelbauer, G. L. (1994) *J. Biol. Chem.* 269, 16449–16454
27. Spudich, E. N., Takahashi, T., and Spudich, J. L. (1989) *Proc. Natl. Acad. Sci. U. S. A.* 86, 7746–7750
28. Dunten, P., and Koshland, D. E., Jr. (1991) *J. Biol. Chem.* 266, 1491–1496
29. Borkovich, D. A., Alex, L. A., and Simon, M. I. (1992) *Proc. Natl. Acad. Sci. U. S. A.* 89, 6756–6760
30. Hanlon, D. W., and Ordal, G. W. (1994) *J. Biol. Chem.* 269, 14038–14046
31. Shapiro, M. J., and Koshland, D. E., Jr. (1994) *J. Biol. Chem.* 269, 11054–11059
32. Ordal, G. W., and Goldman, D. J. (1975) *Science* 189, 892–895
33. Ullah, A. H. J., and Ordal, G. W. (1981) *J. Bacteriol.* 145, 958–965
34. Fisher, C. L., and Pei, G. K. (1996) *BioTechniques* 23, 570–574
35. Smibert, R. M., and Krieg, N. R. (1994) in *Methods for General and Molecular Bacteriology* (Gerhardt, P., Murray, R. G. E., Wood, W. A., and Krieg, N. R., eds) Vol. 1, pp. 603–654, ASM Press, Washington, D. C.
36. Laemmli, U. K. (1970) *Nature* 227, 680–685
37. Laskey, R. A., and Mills, A. D. (1975) *Eur. J. Biochem.* 56, 335–341
38. Kirby, J. R., Niewold, T. B., Maley, S., and Ordal, G. W. (2000) *Mol. Microbiol.* 35, 44–57
39. Thoeike, M. S., Bedale, W. A., Nettleton, D. O., and Ordal, G. W. (1987) *J. Biol. Chem.* 262, 2811–2816
40. Ordal, G. W., Villani, D. P., and Gibson, K. J. (1977) *J. Bacteriol.* 129, 156–165
41. Block, S. M., Segall, J. E., and Berg, H. C. (1983) *J. Bacteriol.* 154, 312–323
42. Nettleton, D. O., and Ordal, G. W. (1991) *J. Bacteriol.* 171, 120–123
43. Boyd, A., and Simon, M. I. (1979) *J. Bacteriol.* 143, 809–815
44. Kim, K. K., Yokota, H., and Kim, S. H. (1999) *Nature* 400, 787–792
45. Muller, J., Scheil, S., Ordal, G. W., and Saxild, H. H. (1997) *Microbiology* 143, 3231–3241