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The role of heterologous receptors in McpB-mediated signalling in *Bacillus subtilis* chemotaxis

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Summary

Asparagine chemotaxis in *Bacillus subtilis* appears to involve two partially redundant adaptation mechanisms: a receptor methylation-independent process that operates at low attractant concentrations and a receptor methylation-dependent process that is required for optimal responses to high concentrations. In order to elucidate these processes, chemotactic responses were assessed for strains expressing methylation-defective mutations in the asparagine receptor, McpB, in which all 10 putative receptors (10del), five receptors (5del) or only the native copy of *mcpB* were deleted. This was done in both the presence and the absence of the methylesterase CheB. We found that: (i) only responses to high concentrations of asparagine were impaired; (ii) the presence of all heterologous receptors fully compensated for this defect, whereas responses progressively worsened as more receptors were taken away; (iii) methyl-group turnover occurred on heterologous receptors after the addition of asparagine, and these methylation changes were required for the restoration of normal swimming behaviour; (iv) in the absence of the methylesterase, the presence of heterologous receptors in some cases caused impaired chemotaxis; and (v) either a certain threshold number of receptors must be present to promote basal CheA activity, or one or more of the receptors missing in the 10del background (but present in the 5del background) is required for establishing basal CheA activity. Taken together, these findings suggest that many or all chemoreceptors work as an ensemble that constitutes a robust chemotaxis system. We propose that the ability of non-McpB receptors to compensate for the methylation-defective McpB mutations involves lateral transmission of the adapted conformational change across the ensemble.

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Introduction

Chemotaxis is the process by which motile bacteria sense their environment and move towards more favourable surroundings. Environmental ligands are sensed upon binding to transmembrane receptors called methyl-accepting chemotaxis proteins (MCPs). *Bacillus subtilis* has 10 putative receptors (Kunst *et al*., 1997). They are: the chemoreceptors McpA, McpB and McpC (Hanlon and Ordal, 1994; Muller *et al*., 1997); the previously identified transducer-like proteins TlpA, TlpB and TlpC (Hanlon and Ordal, 1994; Hanlon *et al*., 1994); the aerotaxis receptor HemAT (Hou *et al*., 2000); and three proteins of unknown function: YfmS, YoaH and YvaQ (Kunst *et al*., 1997). Asparagine chemotaxis is mediated solely through McpB (Hanlon and Ordal, 1994). Adding asparagine to McpB-containing membranes *in vitro* activates an associated autophosphorylating histidine kinase, CheA (Garrity and Ordal, 1997). CheA is associated with the receptor by either of two proteins, CheW and CheV (Hanlon *et al*., 1992a,b; Frederick and Helmann, 1994; Rosario *et al*., 1994). Heightened CheA activity increases the level of the phosphorylated response regulator, CheY-P, which binds to the motor switch to induce a counterclockwise (CCW) or smooth-swimming response (Bischoff *et al*., 1993; Garrity and Ordal, 1997). In the absence of CheY-P, the flagella default to clockwise (CW) rotation, which causes cells to tumble (Bischoff and Ordal, 1991; Fuhrer and Ordal, 1991). Tumbles serve to reorient the bacteria randomly and thereby change the direction of subsequent smooth swims. Net migration through a chemical gradient is achieved in *Escherichia coli* by deviating CheA activity from basal levels to prolong the durations of smooth swims when cells are heading in a beneficial direction (Segall *et al*., 1986) and is presumed to work similarly in *B. subtilis*. Bacteria sense changes in their environment as a function of time (Macnab and Koshland, 1972; Brown and Berg, 1974). Migration through a concentration gradient thus demands that bacteria have a ‘memory’, in the sense that they must be able to determine whether their current environment is better or worse than that sampled previously. Bacteria achieve this feat by using phasic

receptors that reset the system to the original CheA activity after stimulation. This adaptive process allows cells to respond to still further environmental changes. Adaptation to high attractant concentrations in *B. subtilis* involves reversible methylesterifications on certain glutamate residues within a defined methylation consensus sequence (Ahlgren and Ordal, 1983; Kirsh *et al*., 1993a,b). A methyltransferase, CheR, methylates these glutamate residues using *S*-adenosylmethionine as the methyl donor (Springer and Koshland, 1977; Burgess-Cassler *et al*., 1982). A methylesterase, CheB, demethylates the sites, releasing volatile methanol from the cell (Stock and Koshland, 1978; Kirby *et al*., 1997). One methylation site on McpB is encoded as a glutamine in the nascent protein (Hanlon and Ordal, 1994). Such glutamines in enteric bacteria are deamidated by the methylesterase to become glutamates, which are then subject to CheR-dependent methylation (Kehry *et al*., 1983). In *B. subtilis*, receptor deamidation is catalysed by a separate protein called CheD (Kristich and Ordal, 2002). The purpose of deamidation or methylation changes is for the (methylated) state of the receptors to reflect (and thereby compensate for) the current extracellular environment to 'reset' CheA activity to its unstimulated level (Dunten and Koshland, 1991; Borkovich *et al*., 1992). However, the mechanism by which these methylation changes promote adaptation is strikingly different between *B. subtilis* and enteric bacteria. In *E. coli*, receptors are methylated to promote adaptation to positive stimuli and demethylated to promote adaptation to negative stimuli (Goy *et al*., 1977), and so release methanol only to negative stimuli, whereas *B. subtilis* receptors release methanol in response to all stimuli tested (Kirby *et al*., 1997). McpB has been shown to undergo transient net demethylation upon both addition and removal of asparagine (Kirby *et al*., 2000).

Further studies suggest that McpB is selectively methylated at key sites during an asparagine addition and removal time course, and an electrostatic model has been proposed to account for the way in which these selective methylation changes promote adaptation to high asparagine concentrations (Zimmer *et al*., 2000). *B. subtilis* has three chemotaxis proteins not found in *E. coli*: CheC, CheD and CheV (Hanlon *et al*., 1992a,b; Frederick and Helmann, 1994; Rosario *et al*., 1994). CheV has both CheW-like and CheY-like domains. The CheY-like domain accepts phosphoryl groups from CheA (Karatan *et al*., 2001), whereas the CheW-like domain is sufficient for basal CheA activity in the absence of CheW (Karatan *et al*., 2001). CheD has recently been shown to be a glutamine glutamidase that deamidates the nascent glutamine residues within certain methylation consensus sites (Kristich and Ordal, 2002). Receptors in a *cheD* mutant are undermethylated, and such cells exhibit an extreme CW bias (Rosario *et al*., 1995). CheD is absolutely required for McpC- but not McpB-mediated signalling (Kirby *et al*., 2001). The role of CheC remains obscure, but it is related to a family of flagellar switch proteins and may act independently of CheD (Kirby *et al*., 2001). CheC and CheD bind both to each other and to the cytosolic portion of McpB (Rosario *et al*., 1995; Kirby *et al*., 2001). CheC is also known to bind CheA and plays a role in adaptation to high concentrations of the proline analogue azetidine-2-carboxylate (AZC) (Rosario *et al*., 1995; Kirby *et al*., 2001). CheC and CheV also affect receptor methylation (Rosario *et al*., 1995; Rosario and Ordal, 1996; Karatan *et al*., 2001). However, CheC has no apparent effect upon methylesterase activity *per se* (Rosario and Ordal, 1996).

A growing body of evidence suggests that *B. subtilis* has a methylation-independent adaptation system that works at low attractant concentrations. Chemotaxis to AZC and to asparagine in capillary assays was more or less normal at low concentrations and yet severely impaired at high concentrations in the absence of the methylesterase (Kirsh *et al*., 1993b; Kirby *et al*., 1999). Similarly, a methylesterase mutant exhibited an abnormal response to high levels of AZC in tethered cell assays, yet had a more normal response to lower concentrations of AZC (Kirsh *et al*., 1993b).

In order to elucidate what appear to be two distinct yet overlapping adaptation mechanisms, an extensive study was performed on strains expressing mutant McpB proteins (with aspartates substituted at the various sites of methylation) in genetic backgrounds lacking all 10 putative receptors (10del), five receptors (5del) or only the native copy of *mcpB*, both with and without CheB. *E. coli* Tar proteins with aspartates substituted for the sites of methylation were very poorly methylated, suggesting that the altered sites have a fixed negative charge (Shapiro and Koshland, 1994). In *B. subtilis*, a triple aspartate substitution mutant exhibited no detectable methylation in pulse-labelled methylation experiments, and a strain expressing this mutation in the 5del background released no detectable methanol in continuous flow assays (Zimmer *et al*., 2000).

Results

All aspartate substitution mutations are expressed to approximately wild-type levels in all genetic backgrounds

In order to determine whether the observed behavioural phenotypes were not simply attributable to altered expression levels, anti-McpB immunoblots were performed on all strains used in this study. The mutations in the 5del (lacking *mcpA*, *tlpA*, *tlpB*, *mcpC* and the wild-type copy of *mcpB*) background were previously reported to be

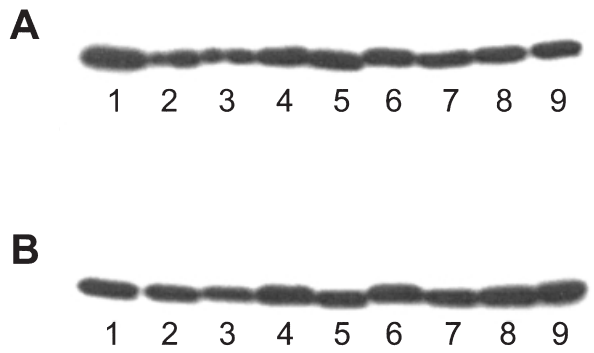

Fig. 1. Western blot analysis of McpB mutations. Experiments were performed as described in *Experimental procedures*. Lane 1 is wild-type McpB in the 5del background. Lanes 2–9 are wild-type McpB, McpB(Q371D), McpB(E630D), McpB(E637D), McpB(Q371D E630D), McpB(Q371D E637D), McpB(E630D E637D) and McpB(Q371D E630D E637D) in the (A) 10del and (B) 10del *cheB* genetic backgrounds.

normally expressed (Zimmer *et al*., 2000). This was also found to be the case for the McpB mutations in all other backgrounds. Immunoblots are shown for the McpB mutations in the 10del (lacking, in addition to the genes in the 5del background, *hemAT*, *yoaH*, *yfmS*, *tlpC* and *yvaQ*) and 10del *cheB* genetic backgrounds (Fig. 1).

The tethered cell response for a methylesterase mutant to low asparagine concentrations is normal

Previous observations showed that the methylesterase-null strain OI2836 exhibited a nearly wild-type response to low concentrations of the proline analogue AZC, yet showed poor chemotaxis to high concentrations of AZC in capillary assays (Kirsh *et al*., 1993b). A similar phenotype was observed in asparagine capillary assays with the methylesterase-null strain OI3319 (Kirby *et al*., 1999). This result suggests that *B. subtilis* is able to adapt to low attractant concentrations in a methylation-independent manner. To find out whether the behavioural response to low concentrations of attractant is indeed normal in the methylesterase mutant, a tethered cell analysis of strain OI2836 was performed to 6.22, 56.0, and 504 μM asparagine (calculated to occupy 10%, 50% and 90% of the McpB proteins respectively) (Fig. 2A). The thick line shows the response to 504 μM asparagine. At this concentration, the strain had a wild-type bias, normal excitation and partial adaptation to the addition of asparagine, yet failed to maintain the adapted state over time as the adapted bias crept up to ≈ 75%. Moreover, upon removal of 504 μM asparagine, there was no true tumble response. Rather, following a delay of ≈ 3 min after asparagine removal, the bias dropped down to prestimulus levels without a period of decreased CCW probability. On the other hand, the response of strain OI2836 to 6.22 μM asparagine (thin line) appeared to be normal. The response to 56.0 μM asparagine (medium line) was intermediate. Thus, the methylesterase is not required for a wild-type response to low asparagine concentrations, which is consistent with the hypothesis that adaptation under these conditions is methylation independent.

Heterologous receptors restore methanol release to a strain expressing the mcpB*(Q371D E630D E637D) allele*

We wanted to explore the possibility that methylation changes on other receptors occur upon stimulation of a mutant McpB that is itself incapable of releasing methanol, similar to what was reported in *Halobacterium salinarum* (Spudich *et al*., 1989). A strain containing the *mcpB*(Q371D E630D E637D) allele in the 5del background exhibited no methanol release in continuous flow assays (Zimmer *et al*., 2000). As adaptation to high asparagine concentrations is, by all accounts, a methylation-dependent process, we decided to test whether het-

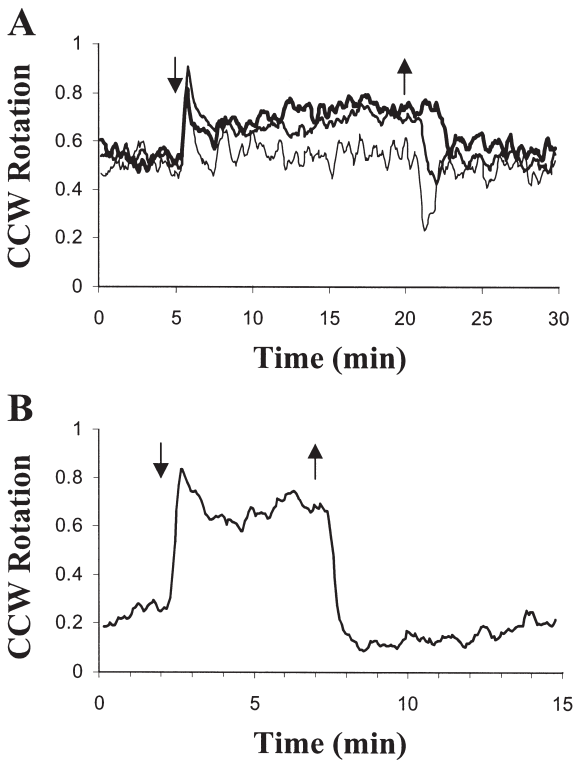

Fig. 2. Tethered cell assays. Experiments were performed as described in *Experimental procedures*.
A. Tethered cell assays on the *cheB* mutant OI2836 to 6.22, 56.0 and 504 μM asparagine (calculated to occupy 10%, 50% and 90% of the McpB proteins respectively). The thick line indicates response to 504 μM asparagine. The medium line indicates response to 56.0 μM asparagine. The thin line indicates response to 6.22 μM asparagine.
B. Tethered cell assay on a strain containing the wild-type *mcpB* allele in the10del background to 504 μM asparagine. Downward arrows indicate the addition of asparagine. Upward arrows indicate asparagine removal.

erologous receptors might undergo methylation changes in response to high concentrations of the McpB-mediated attractant asparagine. To this end, continuous flow assays were performed on a strain expressing the *mcpB*(Q371D E630D E637D) allele in the *mcpB* background. Methanol was indeed released from this strain in response to 504 μM asparagine, suggesting that heterologous receptors undergo methylation changes that may help to promote adaptation (Fig. 3A). Similar methanol release was observed when 504 μM asparagine was added to cells expressing the *mcpB*(Q371D E630D E637D) allele in the (*mcpA mcpB tlpA tlpB*)101::*cat* and *mcpB1*::*cat mcpC4*::*erm* backgrounds (data not shown).

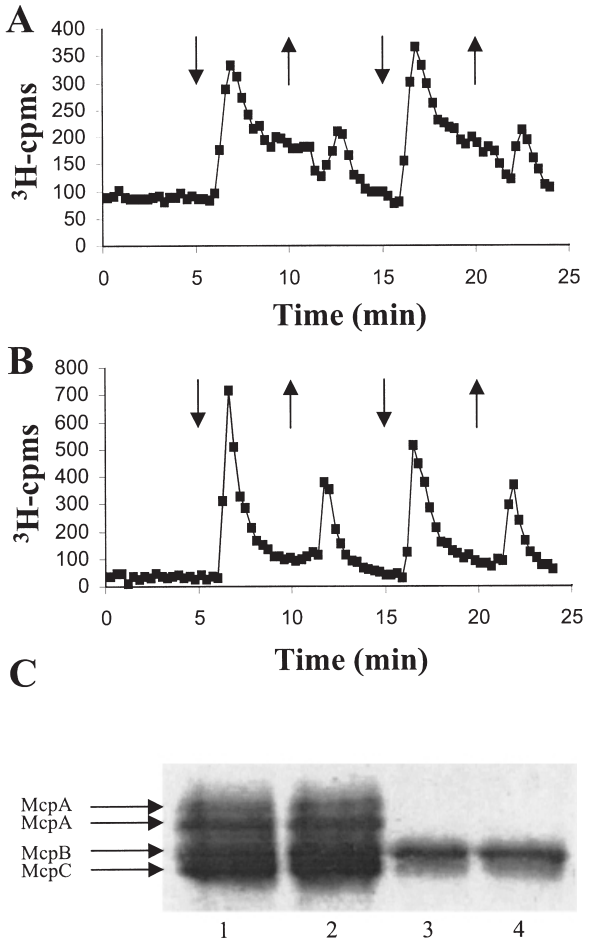

Fig. 3. Continuous flow assays. Experiments were performed as described in *Experimental procedures*.
A. Continuous flow assay on strains containing the *mcpB*(Q371D E630D E637D) allele in the *mcpB* background.
B. Continuous flow assays on strains containing the wild-type *mcpB* allele in the 10del background to 504 μM asparagine. Upward arrows denote asparagine removal.
C. Pulse label methylation experiment on wild-type strain OI1085 versus wild-type *mcpB* expressed in the 10del background. Lanes 1 and 2 contain identically loaded samples of wild-type strain OI1085. Lanes 3 and 4 contain identically loaded samples of strain OI3605 containing the wild-type *mcpB* allele in the 10del background.

Heterologous receptors compensate for the effects of mcpB *aspartate substitutions*

In view of the likelihood that methylation changes, evidenced by methanol release, occur on heterologous receptors in response to the McpB-mediated attractant asparagine, we wanted to know whether these methylation changes facilitated chemotaxis. To this end, capillary assays were performed on strains containing each mutant *mcpB* allele in the *mcpB*, 5del and 10del backgrounds. Strains with the wild-type *mcpB* allele exhibited normal chemotaxis to asparagine in all receptor deletion backgrounds (Fig. 4A). Similarly, strains with the *mcpB*(Q371D) allele appeared to have no chemotactic defect in any background (Fig. 4B). Migration towards high asparagine concentrations was impaired for strains containing all remaining mutant alleles when expressed in the 5del and 10del backgrounds, as evidenced by the decreased accumulation of cells in capillaries (Fig. 4C–H). Thus, the presence of all heterologous receptors appears to compensate completely for chemotactic defects resulting from a failure to methylate McpB properly. Moreover, chemotaxis became progressively more impaired as more heterologous receptors were taken away (Fig. 4C–H). This was most strikingly observed for strains with the *mcpB*(E630D E637D) allele (Fig. 4G). Capillary assay results for strains expressing the mutant *mcpB* alleles in the *mcpB* and 10del backgrounds are summarized in Table 1. In general, a similar trend was observed on swarm plates. All strains expressing the mutant *mcpB* alleles in the *mcpB* background had a wild-type swarm diameter, whereas chemotactic defects only became apparent in the 5del and 10del backgrounds (Fig. 5A). Surprisingly, a strain with the *mcpB*(Q371D E630D) allele actually had a larger swarm diameter in the 10del background than in the *mcpB* background.

Heterologous receptors do not restore wild-type chemotaxis in capillary assays in the absence of the methylesterase

It seems evident that non-McpB receptors enhance asparagine taxis when McpB itself has been mutated. We next wished to test whether it is the methylation changes that take place on these receptors (as opposed to the presence of the receptors *per se*) that promote asparagine taxis involving mutant McpBs in these circumstances. We therefore performed capillary assays to asparagine on strains expressing *mcpB* aspartate mutants in the 10del *cheB*, 5del *cheB* and *mcpB cheB* backgrounds. As receptor deamidation is CheD dependent (Kristich and Ordal, 2002), all unaltered sites are presumed to be methylated in the absence of the methylesterase. Therefore, the *mcpB* mutants in these backgrounds represent all combinations of fixed-charge

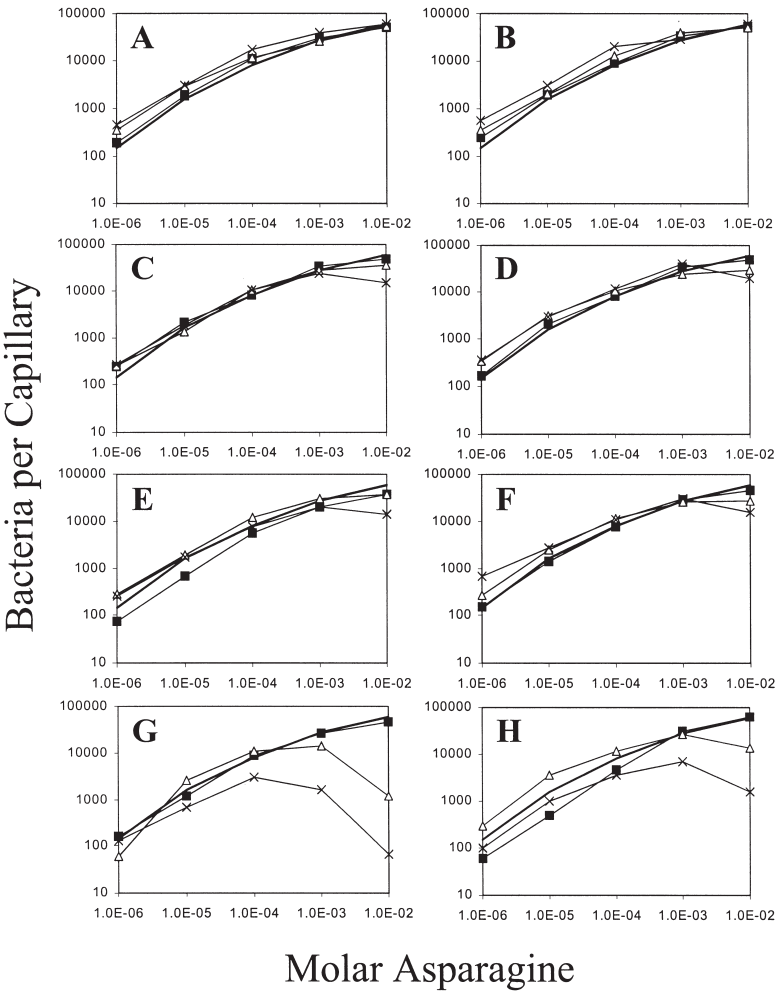

Fig. 4. Capillary assays on strains containing the aspartate substitution mutants in representative *cheB*$^+$ genetic backgrounds. Experiments were performed as described in *Experimental procedures*. For all graphs, the solid squares denote the respective mutants in the *mcpB* background. Open triangles represent the respective mutants in the 5del background. Crosses denote the respective mutants in the 10del background. The thick solid line denotes wild-type strain OI1085 for comparison. Bacterial accumulation is expressed on a logarithmic scale. The expressed *mcpB* alleles are: (A) wild-type *mcpB*; (B) *mcpB*(Q371D); (C) *mcpB*(E630D); (D) *mcpB*(E637D); (E) *mcpB*(Q371D E630D); (F) *mcpB*(Q371D E637D); (G) *mcpB*(E630DE637D); and (H) *mcpB*(Q371D E630D E637D).

states on the receptor. In no instance did the presence of other receptors restore a wild-type response to high asparagine concentrations in the absence of the methylesterase, suggesting that the restoration of chemotaxis is indeed methylation dependent (Fig. 6).

However, we found that chemotaxis to low asparagine concentrations was restored in the presence of heterologous receptors when McpB is in certain fixed methylated conformations (Fig. 6A, D and F). In other fixed methylated conformations, however, the presence of heterologous receptors was not required for wild-type responses to low concentrations of asparagine (Fig. 6B, C, E, G and H). In fact, in some cases, the heterologous receptors even interfered with chemotaxis (Fig. 6C, E and H). Strains with the *mcpB*(Q371D E630D) allele showed wild-type response to low asparagine concentrations and

Table 1. Summary of *cheB*$^+$ capillary assay results.

	methylation site			*mcpB* background		*10del* background	
Expressed *mcpB* allele	371	630	637	low [Asn]	high [Asn]	low [Asn]	high [Asn]
Wild-type *mcpB*	U	U	U	++	++	++	++
mcpB(Q371D)	N	U	U	++	++	++	++
mcpB(E630D)	U	N	U	++	++	++	+
mcpB(E637D)	U	U	N	++	++	++	+
mcpB(Q371D E630D)	N	N	U	++	++	++	+
mcpB(Q371D E637D)	N	U	N	++	++	++	+
mcpB(E630D E637D)	U	N	N	++	++	++	–
mcpB(Q371D E630D E637D)	N	N	N	++	++	++	±

U, site unaltered; N, site always negative; ++, normal response; +, intermediate response; ±, impaired response; –, no response.

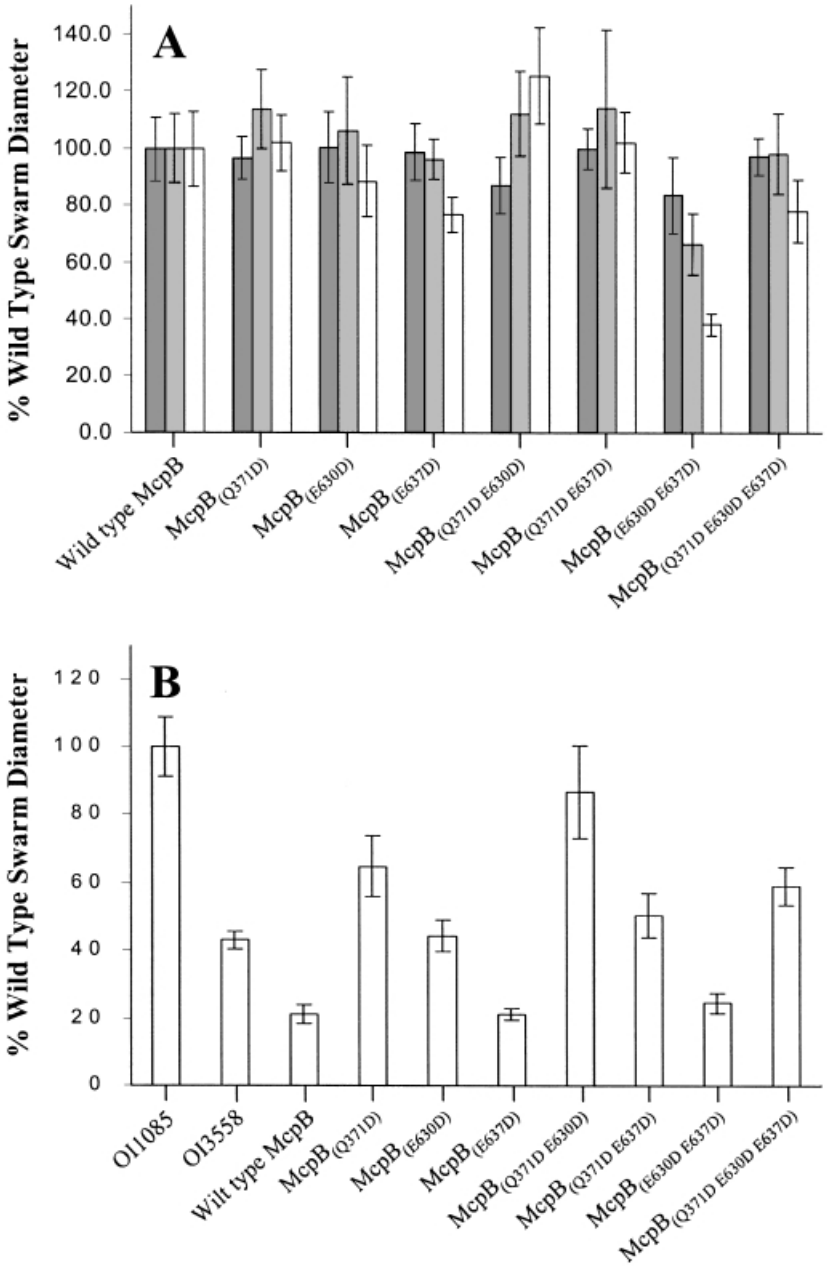

Fig. 5. Swarm assays. Experiments were performed as described in *Experimental procedures*.
A. Swarm assays on strains containing the aspartate substitution mutants in the *mcpB*, 5del and 10del backgrounds. Mutant alleles are as indicated. The dark bars represent strains with the aspartate substitution mutants in the *mcpB* background. The light grey bars denote strains with aspartate substitution mutants in the 5del background, and the white bars denote strains with the aspartate mutants in the 10del background. Mutant swarm diameters are expressed as the percentage relative to the wild-type *mcpB* allele in the isogenic background. The error bars denote standard deviation.
B. Swarm assays on strains containing the aspartate substitution mutants in the 10del *cheB* background. Mutant alleles are as indicated. Swarm diameters are expressed as the percentage relative to wild-type strain OI1085. The error bars denote standard deviation.

nearly normal response to high concentrations (Fig. 6E). Similarly, the swarm diameter of a strain expressing this allele in the 10del *cheB* background appeared to be normal (Fig. 5B). The results for capillary assays for strains expressing the mutant *mcpB* alleles in the *mcpB cheB* and 10del *cheB* backgrounds are summarized in Table 2.

Heterologous receptors are required for normal prestimulus bias

The capillary assay measures chemotaxis in a spatial gradient but gives little information about the rotational behaviour of flagella upon addition and removal of attractant. To explore how heterologous receptors might affect the response of mutant McpBs to asparagine, a tethered cell assay was performed on a strain expressing the wild-type *mcpB* allele in the 10del background. The prestimulus bias for both the wild-type strain OI1085 and a strain containing the wild-type *mcpB* allele in the 5del background is around 58% (Zimmer *et al.*, 2000). However, the bias conferred by the wild-type *mcpB* allele in the 10del background was only 20% (Fig. 2B). Nevertheless, this strain adapted to an approximately wild-type bias

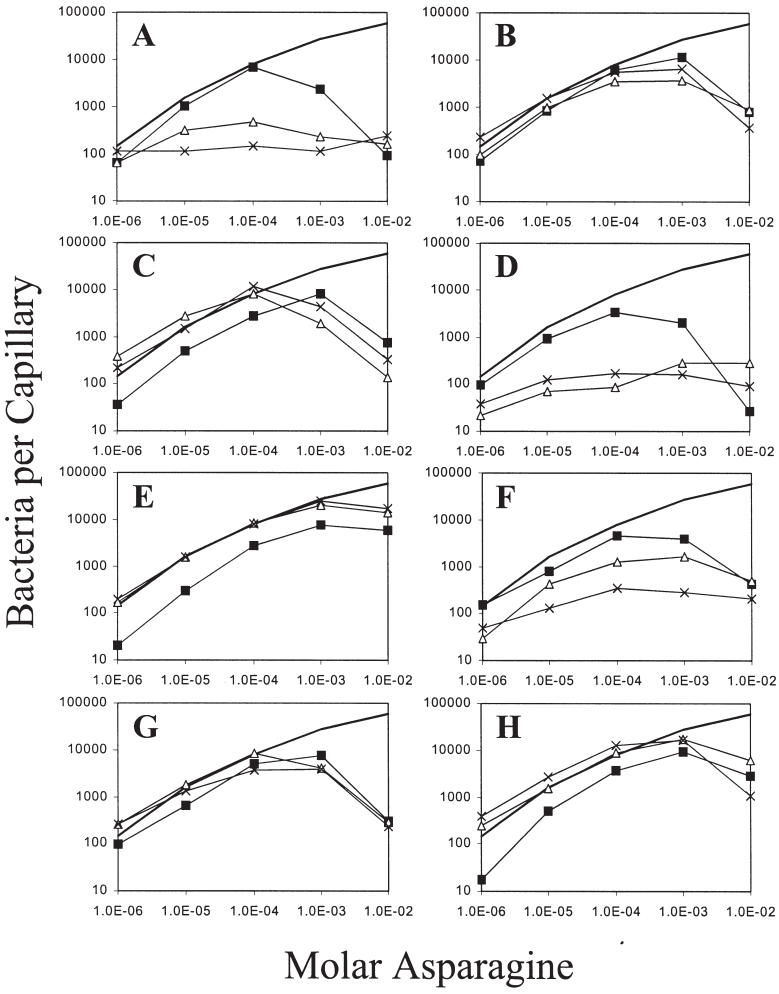

Fig. 6. Capillary assays on strains containing the aspartate substitution mutants in representative *cheB*⁻ genetic backgrounds. Experiments were performed as described in *Experimental procedures*. For all graphs, the solid squares denote the respective mutants in the *mcpB cheB* background. Open triangles represent the respective mutants in the 5del *cheB* background. Crosses denote the respective mutants in the 10del ch*e*B background. The thick solid line denotes wild-type strain OI1085 for comparison. Bacterial accumulation is expressed on a logarithmic scale. The expressed *mcpB* alleles are: (A) wild-type *mcpB*; (B) *mcpB*(Q371D); (C) *mcpB*(E630D); (D) *mcpB*(E637D); (E) *mcpB*(Q371D E630D); (F) *mcpB*(Q371D E637D); (G) *mcpB*(E630D E637D); and (H) *mcpB*(Q371D E630D E637D).

(60–65%) after the addition of asparagine. The altered prestimulus bias of this strain is surprising, as this same mutant showed normal accumulations in capillary assays (Fig. 4A). Therefore, either a certain threshold of total receptors must be present in order to promote basal levels of CheA activity, or one or more of the receptors, TlpC, HemAT, YfmS, YoaH or YvaQ, is specifically required for establishing basal CheA activity.

Heterologous receptors are not required for proper McpB methylation

We wanted to discover the basis of the low bias in the strain with no receptors except wild-type McpB. One possibility by which heterologous receptors might help to establish basal CheA activity is by their facilitating proper methylation changes on wild-type McpB. A similar situa-

Table 2. Summary of *cheB*⁻ capillary assay results.

	methylation site			*mcpB cheB* background		*10del cheB* background	
Expressed *mcpB* allele	371	630	637	low [Asn]	high [Asn]	low [Asn]	high [Asn]
Wild-type *mcpB*	M	M	M	++	–	–	–
mcpB(Q371D)	N	M	M	++	±	++	±
mcpB(E630D)	M	N	M	+	±	++	±
mcpB(E637D)	M	M	N	++	–	–	–
mcpB(Q371D E630D)	N	N	M	+	+	++	+
mcpB(Q371D E637D)	N	M	N	++	–	–	–
mcpB(E630D E637D)	M	N	N	++	–	++	–
mcpB(Q371D E630D E637D)	N	N	N	+	±	++	±

M, site always methylated; N, site always negative; ++, normal response; +, intermediate response; ±, impaired response; –, no response.

tion has been reported for the low abundance *E. coli* receptor Trg, which lacks a conserved C-terminus NWETF methylesterase/methyltransferase docking site (Wu *et al*., 1996; Barnikov *et al*., 1998; Feng *et al*., 1999). To explore this issue further, continuous flow assays and pulse-labelled methylation experiments were performed on cells expressing the wild-type *mcpB* allele in the 10del background. Normal methanol release was observed in response to 504 μM asparagine, indicating that methanol release does not depend upon the presence of heterologous receptors (Fig. 3B). Moreover, pulse label methylation experiments revealed that wild-type McpB from this same background was methylated to approximately wild-type levels (Fig. 3C). This result suggests that McpB itself possesses all the methylesterase/methyltransferase docking sites required for normal methylation.

Discussion

Bacillus subtilis appears to have two partially redundant adaptation systems: a methylation-independent mechanism that operates at low attractant concentrations and a methylation-dependent mechanism that is required for optimal responses to high concentrations. This observation was first made in capillary assays with the *cheB* strain OI2836 and the proline analogue AZC, the response of which is mediated by McpC (Muller *et al*., 1997). Chemotaxis was nearly normal to low AZC concentrations, yet was deficient to high concentrations of AZC (Kirsh *et al*., 1993b). Moreover, this strain only partially adapted to the addition of 10 μM AZC in tethered cell assays, but exhibited more wild-type adaptation in response to the addition of 2.5 μM AZC (Kirsh *et al*., 1993b). Similar results have been seen in capillary assays to the McpB-mediated attractant asparagine by the *cheB* strain OI3319 (Kirby *et al*., 1999). To extend this analysis, tethered cell assays were performed with the *cheB* null strain OI2836 to 6.22, 56.0 and 504 μM asparagine (calculated to occupy 10%, 50% and 90% of the McpB proteins respectively). Consistent with the hypothesis that adaptation to low concentrations of attractant is methylation independent, the response to 6.22 μM asparagine appeared to be normal, whereas the response of tethered cells to 504 μM asparagine was severely defective (Fig. 2A). The response to 56.0 μM asparagine was intermediate, suggesting that there is no absolute concentration 'cut-off' at which the methylation-independent system shuts off and the methylation-dependent system is turned on (Fig. 2A). Rather, there appears to be a broad concentration range over which both systems operate. It is possible that each system may work independently at extremely low and high concentrations. However, it seems more likely that the methylation-independent system always operates and that an auxiliary methylation-dependent system is needed to allow for optimal responses to high asparagine concentrations.

To understand better the functional differences between the two adaptation systems, asparagine capillary assays were performed for a set of strains expressing methylation-defective McpB proteins in the *mcpB*, 5del, and 10del genetic backgrounds (Fig. 4). From these data, several observations can be made. (i) All aspartate substitution mutants in the *mcpB* background showed normal chemotaxis to all concentrations of asparagine (Fig. 4A–H). Thus, heterologous receptors have the ability to mask the chemotactic defects that result from an inability to methylate McpB properly. (ii) Strains containing either the wild-type or the *mcpB*(Q371D) allele exhibited normal responses in all genetic backgrounds (Fig. 4A and B). Therefore, as long as McpB can be properly methylated, the presence of heterologous receptors is superfluous. A fixed negative charge at residue 371 does not appear to affect chemotaxis adversely. (iii) The remaining mutants showed impaired responses to high concentrations of asparagine in the 5del and 10del backgrounds but exhibited normal chemotaxis to low concentrations of asparagine (Fig. 4C–H). Thus, when McpB itself cannot be properly methylated, chemotaxis is only impaired at high asparagine concentrations. This result is consistent with adaptation to low asparagine concentrations being methylation independent. Chemotaxis to low asparagine concentrations did not depend upon the presence of heterologous receptors. (iv) The defective phenotypes were more extreme in the 10del background than in the 5del background. Thus, no one receptor seems to be solely responsible for the restoration of normal chemotaxis to high asparagine concentrations, and perhaps the full complement of heterologous receptors is required to compensate for these defects completely. Generally speaking, a similar trend was observed for these mutants in swarm assays (Fig. 5A). All mutants yielded swarms of approximately wild-type diameter when the full complement of other receptors was present, and the swarm diameter became progressively smaller in the 5del and 10del backgrounds. As adaptation to high asparagine concentrations is methylation dependent, we propose that methylation changes on the heterologous receptors must be required for their normalizing effect on chemotaxis to high concentrations of asparagine. Continuous flow assays were therefore performed on strains containing the *mcpB*(Q371D E630D E637D) allele in the *mcpB* background to test for methanol release from heterologous receptors. Strains expressing the *mcpB*(Q371D E630D E637D) allele in the 5del background exhibited no detectable methanol release (Zimmer *et al*., 2000). However, methanol was released from a strain expressing the *mcpB*(Q371D E630D E637D) allele in the presence of all heterologous receptors (Fig. 3A). Presumably,

this methanol release represents methyl-group turnover on heterologous receptors, although it remains a formal possibility that the presence of the other receptors merely facilitates methyl-group turnover from some cryptic site (or sites) on McpB. Therefore, McpA, McpC, TlpA and TlpB may all undergo methylation changes in response to the McpB-mediated attractant asparagine. To explore this issue further, the *mcpB*(Q371D E630D E637D) allele was placed into both (*mcpA mcpB tlpA tlpB*)101::*cat* and *mcpB mcpC* backgrounds. Methanol was released in response to asparagine in both cases (data not shown). We conclude that methyl-group turnover occurs on McpC and on some combination of McpA, TlpA or TlpB in response to McpB-mediated attractants.

To determine whether these methylation changes on non-McpB receptors were required for the restoration of normal chemotaxis to high concentrations of asparagine, we performed asparagine capillary assays on strains containing the mutant *mcpB* alleles in the *mcpB*, 5del and 10del backgrounds in the absence of the methylesterase. In no case were the heterologous receptors able to compensate for McpB-mediated chemotactic defects when they themselves were unable to undergo proper methylation changes (Fig. 6). Therefore, methylation changes on heterologous receptors are required for their ability to compensate for chemotactic defects resulting from the mutant *mcpB* alleles. We imagine that methylation changes on heterologous receptors somehow 'force' methylation-defective McpB mutants to undergo an adapted conformation (Fig. 7).

Methylation 'cross-talk' among receptors exists in E. coli

The low-abundance chemoreceptor Trg is insufficient to promote a wild-type response to Trg-mediated attractants in the absence of two high-abundance receptors (Silverman and Simon, 1977; Springer *et al*., 1977; Feng *et al*., 1997). This observation has been linked to Trg

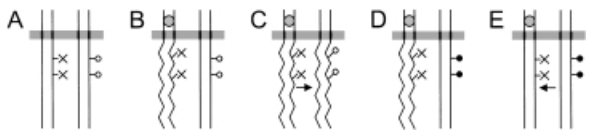

Fig. 7. Model for heterologous receptors compensating for methylation-defective McpB receptors. Each frame depicts a methylation-defective McpB protein (left) next to a wild-type heterologous receptor (right).
A. Receptors in the prestimulus state.
B. Asparagine binds to the mutant McpB and promotes a conformational change that increases CheA activity.
C. This conformational change is transduced laterally to other receptors.
D. The heterologous receptor undergoes methylation changes that promote an adapted state.
E. The conformational change of the adapted state is in turn transduced laterally back to the methylation-defective McpB protein.

lacking a conserved C-terminus NWETF methyltransferase/methylesterase docking site, which makes it poorly methylated unless other receptors containing this motif are present (Sanders and Koshland, 1988; Wu *et al*., 1996; Barnikov *et al*., 1998; 1999; Feng *et al*., 1999). It is thought that the NWETF motif serves to attach CheR to a flexible tether that allows it to contact methylation sites on nearby receptors (Bogonez and Koshland, 1985; Falke *et al*., 1997; Li *et al*., 1997). In the case of *B. subtilis*, however, it is the ability of heterologous receptors themselves to undergo methylation changes that allows them to assist in McpB-mediated chemotaxis. Heterologous receptors are not needed to promote normal methanol release from McpB in response to 504 μM asparagine (Fig. 3B). Similarly, pulse-labelled methylation experiments reveal that wild-type McpB is methylated to approximately normal levels in the absence of other receptors (Fig. 3C). This result suggests that McpB possesses its own binding site (or sites) for the methylesterase and methyltransferase. There is a conserved FKIE motif found at the extreme C-terminus of several *B. subtilis* receptors, including McpB. It is tempting to speculate that this motif may confer a function similar to the NWETF motif in *E. coli*. Unlike *E. coli* receptors, the repeating heptad (a-b-c-d-e-f-g) pattern of McpB – diagnostic for coiled-coils and four-helix bundles – extends the entire length of the C-terminus (Le Moual and Koshland, 1996), suggesting that McpB would lack a flexible tether. An *mcpB* mutant lacking the extreme C-terminus FKIE motif failed to express (C. Kristich and G. Ordal, unpublished).

It is possible that the mechanism of methylation-independent adaptation may involve the regulation of higher order receptor complexes. *E. coli* receptors are clustered at the cell poles, suggesting that the receptors might function as an aggregate array (Maddock and Shapiro, 1993). Duke and Bray (1999) have proposed that lateral signal transmission across such an ensemble could account for the broad range of attractant concentrations to which bacteria respond. Experiments by Liu *et al*. (1997) in *Salmonella enterica* serovar Typhimurium showed that a soluble C-terminal fragment of the aspartate receptor Tar forms an *in vitro* complex with CheW and CheA with an approximate ratio of 7:2:1 (Tarc-term–CheW–CheA), confirming the existence of a higher order receptor complex. The Bray laboratory has constructed three-dimensional models of the chemotaxis proteins based on known crystal structures and pieced them together manually into a putative higher order complex (Shimizu *et al*., 2000).

They concluded that the putative complex consists of repeating hexagonal units with a stoichiometry of 2:1:1 (receptor–CheW–CheA) in an infinite array (Shimizu *et al*., 2000). McpB proteins of *B. subtilis* are also clustered at the cell poles (Kirby *et al*., 2000). Given the high degree

of similarity among bacterial chemoreceptors (Le Moual and Koshland, 1996), and the fact that *B. subtilis* responds to a similarly broad range of attractant concentrations (Kirby *et al*., 2000), it seems very likely that *B. subtilis* chemoreceptors also act as an ensemble. Moreover, given that (i) signal amplification to low attractant concentrations is thought to involve lateral signal transduction across the receptor array, and (ii) *B. subtilis* can adapt to low concentrations of attractant in a methylation-independent manner, it seems that methylation-independent adaptation involves the regulation of the receptor complex.

An interesting observation is that, for *cheB* strains containing the wild-type *mcpB*, *mcpB*(E637D) or *mcpB*(Q371D E637D) allele, heterologous receptors were required for a response even to low concentrations of asparagine (Fig. 6A, D and F). The remaining mutants, however, taxed more or less normally to low concentrations of asparagine in the absence of heterologous receptors (Fig. 6B, C, E, G and H). In fact, in some cases, heterologous receptors even interfered with chemotaxis (Fig. 6C, E and H). This finding implies that the methylation-independent adaptation system requires certain fixed methylated states in order to operate effectively. Fixed charges, i.e. glutamine (uncharged and thought to be similar to methyl glutamate) or glutamate (charged) states, on the *E. coli* receptor Tsr affect ternary Tsr–CheW–CheA complexes in the sense that the mutant receptors containing multiple glutamine residues are much more susceptible to inhibition of the associated CheA kinase by the attractant aspartate than are receptors containing multiple glutamate residues (Li and Weis, 2000). It is not hard to imagine that these differences result from the structure of the CheA–CheW–receptor complex. We speculate that a default methylation state of McpB facilitates optimal packing interactions that allow for methylation-independent communication between receptors.

We imagine that this effect is caused by lateral transmission of ligand-induced signals across the receptor array in a manner similar to that proposed by Duke and Bray (1999). This interpretation requires that receptor packing interactions are influenced by certain methylated receptor conformations and suggests that the degree to which signals are spread laterally might be regulated. The observation that heterologous receptors make chemotaxis to low concentrations of asparagine possible in some instances (Fig. 6A, D and F) suggests that McpB in these fixed methylated states does not assemble well into higher order complexes and must interact with heterologous receptors for lateral signal transmission to occur. In other instances, specifically for strains expressing *mcpB* mutants with the 630 aspartate substitution, heterologous receptors impaired chemotaxis (Fig. 6C, E and H). This observation suggests that residue 630 is negative when McpB is optimized to assemble into a higher order complex. Heterologous receptors might negatively affect these optimized interactions when they themselves cannot undergo methylation changes. McpB is thought to be methylated at residue 637 and unmethylated at positions 371 and 630 when adapted to high asparagine concentrations (Zimmer *et al*., 2000). This methylation state is presumably mimicked in strains with the *mcpB*(Q371D E630D) mutant in the *cheB* backgrounds. Thus, it appears to be significant that cells expressing this protein in the *cheB* mutants are the most nearly normal in both capillary (Fig. 6E) and swarm assays (Fig. 5B). The data presented in this paper suggest that an inherent interdependence among receptors allows for robust chemotaxis, in the sense that chemotaxis is effective independent of changes in input (i.e. mutational changes in the receptor). This situation might explain why a strain with the wild-type *mcpB* allele in the 10del background had a very low (≈ 20%) prestimulus bias (Fig. 2B). Either a certain threshold number of receptors must be present to promote basal CheA activity or one or more of the receptors missing in the 10del background but present in the 5del background is required for establishing basal CheA activity.

In short, we believe that the evidence presented in this article underscores the likelihood that many or all of the chemoreceptors act as an ensemble that helps to constitute a robust chemotaxis system. In instances in which McpB is wild type, this robustness is 'in reserve' as, even when all other receptors are missing, McpB still mediates wild-type responses to asparagine (Fig. 4). However, when McpB cannot be methylated properly, heterologous receptors restore chemotaxis to high asparagine concentrations. This ability to assist in McpB-mediated chemotaxis to high asparagine concentrations depends on the ability of these heterologous receptors themselves to undergo methylation changes. We imagine that these changes allow methylation-defective McpB molecules to undergo the conformational changes needed to bring about effective chemotaxis (Fig. 7). In the absence of the methylesterase, the presence of heterologous receptors impeded chemotaxis in some cases, whereas in other cases, they were required for it – depending on the methylation state of McpB (Fig. 6). This evidence strongly suggests that receptor-to-receptor contacts laterally transduce information among heterologous receptors in a functional signalling array.

Experimental procedures

Bacterial strains and plasmids

All bacterial strains involved in this study have the mutant or wild-type *mcpB* gene placed into the *amyE* locus under the

control of the native promoter with selection for spectinomycin (spec) resistance. The strains were ultimately derived from the chemotactically wild-type strain OI1085 (Ullah and Ordal, 1981). The 10del genetic background is (*mcpA mcpB tlpA tlpB*)101::*cat mcpC4*::*erm tlpC*::*cat hemAT*::*erm yfmS*::*erm yoaH*::*erm yvaQ*::*erm* (Hou *et al*., 2000), where *cat* refers to chloramphenicol resistance and *erm* to erythromycin resistance. The 10del *cheB* background is (*mcpA mcpB tlpA tlpB*)101::*cat mcpC4*::*erm tlpC*::*cat hemAT*::*erm yfmS*::*erm yoaH*::*erm yvaQ*::*erm cheB8*. The 5del genetic background is (*mcpA mcpB tlpA tlpB*)101::*cat mcpC4*::*erm*. The 5del *cheB* genetic background is (*mcpA mcpB tlpA tlpB*)101::*cat mcpC4*::*erm cheB7*. The *mcpB* strain is *mcpB1*::*cat* (Hanlon and Ordal, 1994). The *mcpB cheB* strain is *mcpB1*::*cat cheB7*. Plasmids used in the construction of these strains are pBluescript SK– (Stratagene), the *E. coli*/*B. subtilis* shuttle vector pEB112 (Leonhardt and Alonso, 1988), the erm-resistant gene inactivation vector pHV501 (Vagner *et al*., 1998) and a modified version of the *amyE* integration vector pDG1730 (Guerout-Fleury *et al*., 1996; Kristich and Ordal, 2002). A complete listing of all strains and plasmids used in this study may be found at the following website: http://www.life.uiuc.edu/biochem/f_ordal.html. All plasmids were propagated in *E. coli* strainTG-1 (Amersham Pharmacia Biotech).

Chemicals, solutions and growth media

L-[methyl-^{3}H]-methionine (80–85 Ci mmol^{-1}) was purchased from Amersham. All other chemicals were reagent grade. Luria–Bertani (LB) medium is 1% tryptone, 0.5% yeast extract and 1% NaCl.

Tryptone (TB) medium is LB without yeast extract. Chemotaxis buffer (CB) is 0.1 mM EDTA, 50 μM $CaCl_2$, 0.05% glycerol, 5 mM sodium lactate, 0.3 mM ammonium sulphate and 20 mM potassium phosphate, pH 7.0 (Ordal and Goldman, 1975). Protoplast buffer (PB) is 20% sucrose, 25 mM potassium phosphate, 10 mM $MgCl_2$, 30 mM sodium lactate and 1.0 mM EDTA, pH 7.0 (Ullah and Ordal, 1981).

Minimal medium is 50 mM potassium phosphate, 1 mM $(NH_4)_2SO_4$, 1.2 mM $MgCl_2$, 140 μM $CaCl_2$, 10 μM $MnCl_2$, with 50 μg ml^{-1} required amino acids and 20 mM sorbitol, pH 7.0 (Ordal and Goldman, 1975).

Construction of mcpB*-expressing strains*

The construction of all *mcpB* aspartate alleles has been described previously (Zimmer *et al*., 2000). The various *mcpB* alleles were crossed into the *amyE* locus of strains OI3056, OI3557, OI3180, OI3182, OI3545 and OI3558 by linearizing the respective pONG subclones with *Aat*II, transforming into the above strains and selecting for Cm^R Spec^R Amy$^-$ (Smibert and Krieg, 1994). The OI3180 and OI3182 strains were then crossed with OI3280 chromosomal DNA, selecting for Erm^R colonies to place the mutant *mcpB* alleles into (*mcpA mcpB tlpA tlpB*)101::*cat mcpC4*::*erm* or (*mcpA mcpB tlpA tlpB*)101::*cat mcpC4*::*erm cheB7* backgrounds respectively. Expression of all *mcpB* alleles was confirmed by immunoblot analysis (Zimmer *et al*., 2000).

Strain OI3557 was made by crossing strain OI2715 with chromosomal DNA from strain OI3056 and selecting for Cm^R. Strain OI3558 was made in a manner parallel to the construction of the 10del strain OI3545 (Hou *et al*., 2000). Briefly, strains with individual *mcpC*::*erm*, *hemAT*::*erm*, *yfmS*::*erm*, *yoaH*::*erm* and *yvaQ*::*erm* mutations were constructed using internal polymerase chain reaction (PCR) gene fragments cloned into the plasmid pHV501 (Vagner *et al*., 1998) and integrated into the chromosome of strain OI1085 with selection for Erm^R. These separate mutations were then combined into a single strain by introducing nearby nutritional markers via co-transformation with pEB112 (Leonhardt and Alonso, 1988), selection for Kn^R, subsequent segregation of the plasmid and co-transduction of the respective inactivated receptor genes with selection for the prototrophy confirmed by the nearby nutritional marker. The *cheB8*::*cat* mutation was introduced by transformation with selection for Cm^R followed by introduction of the *thrC*::*spc* mutation by transformation with selection for Spc^R. The (*mcpA mcpB tlpA tlpB*)101::*cat* mutation was then introduced by PBS1 transduction with selection for Thr$^+$. Finally, the *tlpC*::*kn* mutation was introduced by PBS1 transduction with selection for Kn^R to create the final (*mcpA mcpB tlpA tlpB*)101::*cat mcpC4*::*erm tlpC*::*kn hemAT*::*erm yfmS*::*erm yoaH*::*erm yvaQ*::*erm cheB8*::*cat* strain.

Western blot analysis of McpB mutations

Cells were treated as outlined for the pulse-labelled methylation experiments except that no L-[methyl-^{3}H]-methionine was added. Western analysis of *mcpB* mutants was performed as described previously (Kirby *et al*., 2000). Antigen-antibody was detected using Supersignal (Pierce). Visualization required a 5 s to 1 min exposure of Kodak X-Omat AR film.

Pulse-labelled methylation experiments

Experiments were performed as described previously (Ullah and Ordal, 1981). 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 PB with 250 μg ml^{-1} chloramphenicol and resuspended to A_{600} = 1.0 in 3 ml of PB with 250 μg ml^{-1} chloramphenicol and 4 mg l^{-1} lysozyme. Cells were shaken at 250 r.p.m. at 37°C for 20 min after the addition of 150 μl of 1 μM L-[methyl-^{3}H]-methionine. Aliquots (1 ml) were removed and flash-frozen in dry ice–acetone baths. Samples were then thawed and centrifuged at 3000 *g* for 45 min at 4°C. Pelleted membranes were resuspended in 100 μl of 4× SDS solubilizer, boiled for 7 min and electrophoresed at 15 V cm^{-1} through 10% SDS–polyacrylamide gels, pH 8.8 (Laemmli, 1970). Gels were treated for fluorography as described previously (Laskey and Mills, 1975).

Continuous flow assay

The continuous flow assay has been described previously (Thoelke *et al*., 1987). Briefly, a 100 μl aliquot 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 three times in CB with 250 μg ml^{-1} chloramphenicol and

resuspended to A_{600} = 1.0 in 3 ml of CB with 250 μg ml^{-1} chloramphenicol. Cells were shaken at 250 r.p.m. at 37°C for 20 min after the addition of 150 μl of 1 μM L-[methyl-^{3}H]-methionine. Radiolabelled 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^{-1}. Cells were pretreated with asparagine in CB with 250 μg ml^{-1} chloramphenicol for 4 min and then switched back to CB with chloramphenicol for 6 min before the assay was initiated by collecting 0.3 min fractions. Asparagine (504 μM) 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. A concentration of 504 μM asparagine is predicted to bind 90% of the McpB proteins based upon experimentally determined K_d values (Ordal *et al*., 1977).

Swarm assay for chemotaxis

Mutants were spotted onto semi-solid agar plates (TB with 0.27% agar). As the bacteria metabolize nutrients, a chemical gradient is established, resulting in the formation of a ring as the bacteria tax outwards. To mitigate variability in swarm diameter, two colonies of a control strain expressing the wild-type *mcpB* allele in an isogenic background were also spotted on every plate. The data represent the averaged swarm diameters of 20 colonies, with the mutant swarm diameters expressed as a percentage relative to the control strain (defined as 100%).

Capillary assay for chemotaxis

The capillary assay for chemotaxis in *B. subtilis* has been described previously (Ordal and Goldman, 1975). Strains were grown overnight at 30°C on TBAB plates. Cells were taken off the plate and resuspended in 0.5 ml of minimal media. The suspension was then diluted to A_{600} = 0.014 in 5 ml of minimal media and grown at 37°C with vigorous shaking (250 r.p.m.) for 5.5–6.0 h. Fifteen minutes before harvesting, 50 μl of a 5% glycerol–0.5 M sodium lactate solution was added to the culture. The culture was washed in CB by vacuum filtration (nitrocellulose filter) and resuspended from the filter into CB. Cells were then diluted to A_{600} = 0.001 in CB and used in assays in which the capillaries contained CB or CB plus asparagine. After 30 min, the contents of the capillaries were squirted into top agar, spread over solid agar plates (TB with 1.5% agar), and the resulting colonies were counted the next day.

All experiments were performed twice on separate days, each time in duplicate, to ensure reproducibility of the data.

Tethered cell assay for chemotaxis

The tethered cell assay has been described previously (Kirby *et al*., 2000). Strains were grown on TBAB plates overnight in a 30°C wet incubator. Cells were taken off the plate and suspended in minimal media. The suspension was then diluted to A_{600} = 0.014 in 25 ml of minimal media and grown at 37°C with vigorous shaking (250 r.p.m.) for 4.5 h. Fifteen minutes before harvesting, 200 μl of a 5% glycerol–0.5 M sodium lactate solution was added to the culture. The cells were blended at full speed in a Waring blender for 10 s in order to shear the flagella. A 250 μl aliquot of blended cells was placed onto a glass coverslip preincubated with 10 μl of anti-flagella antibody and incubated for 15 min. The coverslip was then inverted and placed as the ceiling of a laminar flow chamber to be observed by phase-contrast microscopy in a system similar to those described previously (Block *et al*., 1983; Kirsh *et al*., 1993b). CB with or without asparagine was pumped through the laminar flow chamber at a constant rate of 1 ml min^{-1}. Rotating cells were videotaped and analysed by a Hobson Tracker, Bacterial Edition (Hobson Tracking Systems), which generates text files containing continuous-time rotational data. Programs written with MATLAB software (The Mathworks) processed the data. For each cell, the continuous-time rotational data were converted to discrete-time data with a step size of 0.1 s. All the data points within a 4 s window were then averaged to generate a data set with a step size of 4 s that contained the probability of CCW rotation (smooth-swimming behaviour). The data for all cells in the sample population were then pooled and averaged, and the resulting data were smoothed over a 5 data point window to generate the final graph. The asparagine concentrations used in this study were 6.22, 56.0 and 504 μM (calculated to occupy 10%, 50% and 90% of the McpB proteins respectively) (Ordal *et al*., 1977).

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