Molecular Microbiology (2004) **53**(4), 1051–1063 doi:10.1111/j.1365-2958.2004.04176.x

Targeting of the chemotaxis methylesterase/deamidase CheB to the polar receptor–kinase cluster in an *Escherichia coli* cell

Satomi Banno, Daisuke Shiomi,† Michio Homma and Ikuro Kawagishi*

Division of Biological Science, Graduate School of Science, Nagoya University, Chikusa-ku, Nagoya 464-8602, Japan.

Summary

Chemotactic adaptation to persisting stimulation involves reversible methylation of the chemoreceptors that form complexes with the histidine kinase CheA at a cell pole. The methyltransferase CheR targets to the C-terminal NWETF sequence of the chemoreceptor. In contrast, localization of the methylesterase CheB is largely unknown, although regulation of its activity via phosphorylation is central to adaptation. In this study, green fluorescent protein was fused to full-length CheB or its various parts: the N-terminal regulatory domain (N), the C-terminal catalytic domain (C) and the linker (L). The full-length and NL fusions and, to a lesser extent, the LC fusion localized to a pole. Deletion of the P2 domain from CheA abolished polar localization of the full-length and NL fusions, but did not affect that of the LC fusion. Pull-down assays demonstrated that the NL fragment, but not the LC fragment, binds to the P2 fragment of CheA. These results indicate that binding of the NL domain to the P2 domain targets CheB to the polar signalling complex. The LC fusion, like the chemoreceptor, partially localized in the absence of CheA, suggesting that the LC domain may interact with its substrate sites, either as part of the protein or as a proteolytic fragment.

Introduction

In many sensory systems, higher order interactions between multiple protein components are involved in signal transduction. Such interactions might be spatially regulated. Even in bacteria, despite their small sizes, at least some signalling components localize to subcellular compartments, and such localization seems to play critical roles in signal transduction (Lybarger and Maddock, 2001; Shapiro *et al*., 2002). Therefore, it is of vital importance to know subcellular localization and regulated interplays between signalling components to understand how the whole system works to receive signals, to process information and to produce output.

The chemotactic behaviour of *Escherichia coli* (Manson, 1992; Stock and Surette, 1996; Falke *et al*., 1997; Armitage, 1999), movement towards a more favourable environment, is well suited to studying these issues as all signalling components have been characterized in detail. Intracellular chemotactic signals, which are produced by transmembrane chemoreceptors/transducers (also known as methyl-accepting chemotaxis proteins, MCPs) in response to extracellular stimuli, are processed by a His-Asp phosphorelay system (also known as a two-component regulatory system) that is used in a wide variety of signal transduction systems among eubacteria as well as archaea, yeasts, fungi and higher plants (Mizuno, 1998; West and Stock, 2001). An unliganded chemoreceptor (MCP) stimulates the histidine kinase CheA, which autophosphorylates at His-48 and donates the phosphate group to the response regulators, CheY (at Asp-57) and CheB (at Asp-56). Phospho-CheY binds to the flagellar motor to induce clockwise (CW) rotation, whereas the motor rotates counterclockwise (CCW) when phospho-CheY is not bound. Attractant binding inhibits the CheA activity to reduce the probability of CW motor rotation and hence tumbling of the cell. Phosphorylation of the methylesterase (MEase)/deamidase (DAase) CheB enhances its activity: it catalyses demethylation/deamidation of MCPs. Therefore, attractant binding decreases the CheB activity and hence promotes methylation of an MCP, which is catalysed by the methyltransferase (MTase) CheR. Increased receptor methylation reactivates CheA to counteract the initial response. This negative feedback regulation (termed adaptation) is essential for chemotaxis.

The cytoplasmic domain of an MCP has four or five potentially methylatable glutamate residues located in two separate helices (Le Moual and Koshland, 1996; Danielson *et al*., 1997); hence, the receptors are named MCPs. The MTase CheR transfers a methyl group from

Accepted 14 April, 2004. *For correspondence. E-mail i45406a@cc.nagoya-u.ac.jp; Tel. (+81) 52 789 2993; Fax (+81) 52 789 3001. †Present address: Microbiology and Molecular Genetics, University of Texas Medical School, 6431 Fannin, Houston, TX 7730, USA.

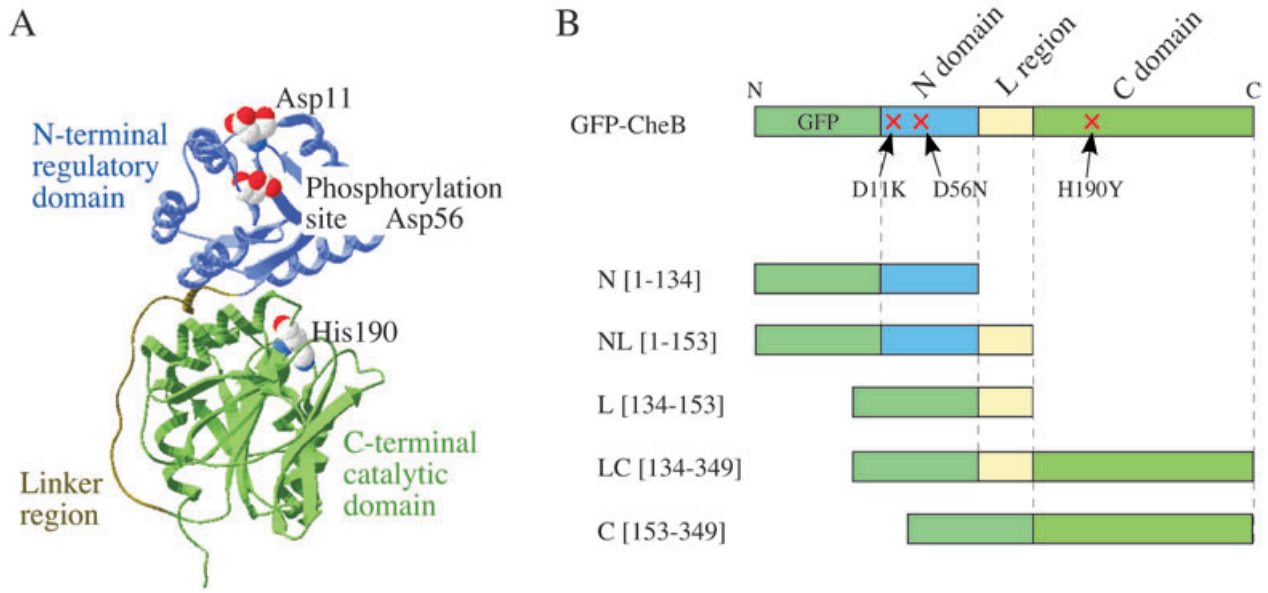

Fig. 1. The GFP–CheB fusion protein and its derivatives.
A. The three-dimensional structure of *Salmonella typhimurium* CheB (Djordjevic *et al*., 1998). The N-terminal regulatory domain (N) is shown in blue, the C-terminal catalytic domain (C) in green and the linker (L) in yellow. Residues mutated in this study are indicated: Asp-11, the active site; Asp-56, the phosphorylation site; and His-190, the catalytic site.
B. The GFP–CheB fusion protein and its derivatives constructed in this study. The residues that are mutated (D11K, D56N and H190Y) are marked with a cross. For CheB fragments fused to GFP, residue numbers are indicated in brackets.

S-adenosyl methionine (AdoMet) to one of the glutamate residues, and the MEase CheB hydrolyses the methyl-ester bond of a methylated glutamate residue. Some of the methylation sites of a newly synthesized chemoreceptor are glutamine residues that are converted to methylatable glutamate residues by the DAase activity of CheB. CheB consists of an N-terminal regulatory domain (N) and a C-terminal catalytic domain (C) that are connected by a flexible linker (L) (Fig. 1) (Anand *et al*., 1998). In the unphosphorylated state, the N domain inhibits the MEase/DAase activity by interacting with the C domain. When phosphorylated, CheB undergoes a conformational change to derepress the MEase/DAase activity (Anand *et al*., 1998). Unlike CheB, the MTase CheR is not regulated by covalent modification. An increase in the concentration of phospho-CheB as a result of the activation of CheA promotes the demethylation of MCP, resulting in adaptation. In contrast, a decrease in the concentration of phospho-CheB causes a net increase in receptor methylation catalysed by CheR.

MCP, CheA and the adaptor protein CheW form a stable ternary complex (Gegner *et al*., 1992; Schuster *et al*., 1993) that localizes at a cell pole (Maddock and Shapiro, 1993; Skidmore *et al*., 2000; Sourjik and Berg, 2000). Such polar localization and/or clustering of the receptor–kinase complexes has been proposed to play a critical role in signal amplification (Bray *et al*., 1998; Duke and Bray, 1999). The first indication of significant co-operativity, or gain, between MCPs was provided by *in vitro* studies (Bornhorst and Falke, 2000; Li and Weis, 2000). *In vivo*, chemically synthesized multivalent ligands induce attractant responses with lower thresholds than corresponding monovalent ligands (Gestwicki *et al*., 2000; Gestwicki and Kiessling, 2002). Recent analyses using fluorescence resonance energy transfer suggested that much of the gain occurs at the receptor end of the signalling pathway (Sourjik and Berg, 2002). The cytoplasmic fragment of the serine chemoreceptor Tsr crystallizes with a hexamer unit of a trimer of dimers (Kim *et al*., 1999). Such trimers of MCP dimers have been proposed to assemble into a lattice-like matrix (Shimizu *et al*., 2000; Kim *et al*., 2002). Genetic analyses support the putative contacts among three dimers, and chemical cross-linking assays detected interaction between Tar and Tsr (Ames *et al*., 2002). *In vivo* disulphide cross-linking assays demonstrated that MCP dimers interact with each other and suggest that attractant binding may alter relative positions or trajectories of MCP dimers that might form a trimer unit (Homma *et al*., 2004).

The receptor-modifying enzymes, CheR and CheB, might also be recruited to the polar receptor–kinase cluster. Indeed, the β-subdomain of the MTase CheR binds to the C-terminal pentapeptide sequence (NWETF) of high-abundance MCPs (i.e. Tsr and Tar) (Wu *et al*., 1996; Djordjevic and Stock, 1998; Okumura *et al*., 1998; Shiomi *et al*., 2000), and this binding is essential for the polar localization of CheR (Shiomi *et al*., 2002), increasing the

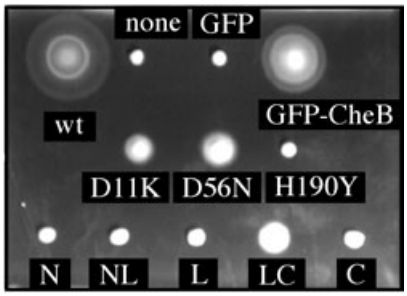

Fig. 2. Swarming ability of RP4953 (ΔCheB) cells carrying the vector pBAD24 (labelled none) or one of its derivatives encoding GFP (labelled GFP) or wild-type (labelled GFP–CheB), mutant (labelled D11K, D56N or H190Y) or truncated (labelled N, NL, L, LC or C) versions of GFP–CheB. RP437 (Che^+) cells carrying the vector pBAD24 (labelled wt) were used as a positive control. Fresh overnight cultures (2 μl each) were spotted on tryptone semi-solid agar supplemented with 50 μg ml^{-1} ampicillin and 1 mM arabinose. The resulting plate was incubated at 30°C for 7 h.

concentration of CheR around MCPs. Barnakov *et al.* (2001; 2002) reported that CheB also binds to the same sequence albeit with much lower affinity. However, localization of the MEase/DAase CheB is largely unknown although it plays a central role in adaptation and is also suggested to be involved in signal amplification (Barkai *et al.*, 2001; Sourjik and Berg, 2002).

In this study, we examined subcellular localization of CheB. Observation of green fluorescence protein (GFP) fused to CheB demonstrated that CheB localizes to a cell pole in the presence of an MCP. The GFP fusion protein with the N-terminal domain and the linker (NL) and, to a lesser extent, that with the linker and the C-terminal domain (LC) localized to a pole. Neither the full-length nor the NL fusion localized to poles of cells lacking the P2 domain of CheA, whereas Tar–GFP localized normally, suggesting that CheB targets to the P2 domain of CheA. In contrast, the LC fusion did not bind to the P2 domain and, even in the absence of CheA, it localized partially to a pole, suggesting that the C domain by itself can target to MCPs and raising the possibility that the methylation helices of MCPs might serve as second targets of CheB localization.

Results

GFP-CheB localizes to a cell pole in the presence of the chemoreceptor

To observe the localization of CheB, we constructed the plasmid pDS901 encoding GFP fused to wild-type CheB (Fig. 1). Mutations were introduced into the *cheB* coding region (Fig. 1): the substitution of Lys for Asp-11 (D11K) of the regulatory domain results in a constitutive active phenotype (pDS902); the substitution of Asn for the phosphorylated site Asp-56 (D56N) abolishes the phosphorylation of CheB (pDS904); and the substitution of Tyr for His-190 (H190Y) of the catalytic centre eliminates the MEase/DAase activity (pDS906) (Djordjevic *et al.*, 1998).

RP4953 (Δ*cheB*) cells expressing wild-type GFP–CheB swarmed in tryptone semi-solid agar slightly more slowly than RP437 (wild-type for chemotaxis) cells (Fig. 2), indicating that the fusion protein is functional at least to considerable extent. Introduction of the H190Y mutation abolished chemotaxis, whereas cells expressing any other mutant version of GFP–CheB showed chemotaxis, although they spread much more slowly than cells expressing wild-type GFP–CheB. Immunoblotting with anti-GFP antibody revealed that wild-type and mutant versions of GFP–CheB were expressed at similar levels except that the H190Y mutant was expressed at a significantly lower level (Fig. 3). All the GFP–CheB proteins were detected by antibody raised against *Salmonella typhimurium* CheB (data not shown).

We then examined subcellular localization of the wild-type and mutant GFP–CheB proteins. Whereas GFP was distributed throughout the cell, all the GFP–CheB derivatives localized to a cell pole (Fig. 4). To avoid a relatively high cell-to-cell variation occasionally seen for transcrip-

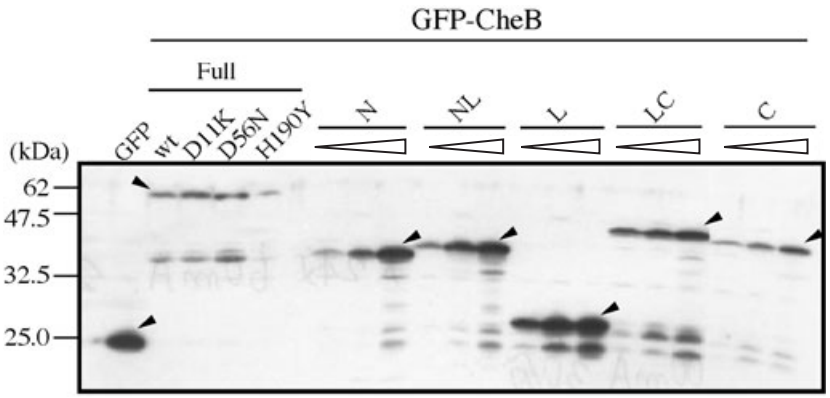

Fig. 3. Expression levels of wild-type, mutant and truncated versions of GFP–CheB in RP4953 (ΔCheB). GFP (pDS900) and full-length GFP–CheB proteins [wt (wild type, pDS901), D11K (pDS902), D56N (pDS904) and H190Y (pDS906)] were expressed from pBAD24 derivatives with 1 mM arabinose. The truncated versions of GFP–CheB were expressed, with increasing levels (denoted by triangles), from pBAD24 derivatives (N, pSB111; NL, pSB121; L, pSB131; LC, pSB141; C, pSB151) with 1 or 5 mM arabinose or from pTrcHisC derivatives (N, pSB211; NL, pSB221; L, pSB231; LC, pSB241; C, pSB251) without an inducer. These proteins were detected by immunoblotting with anti-GFP antibody. Bands for GFP, various versions GFP–CheB fusions are indicated with arrowheads.

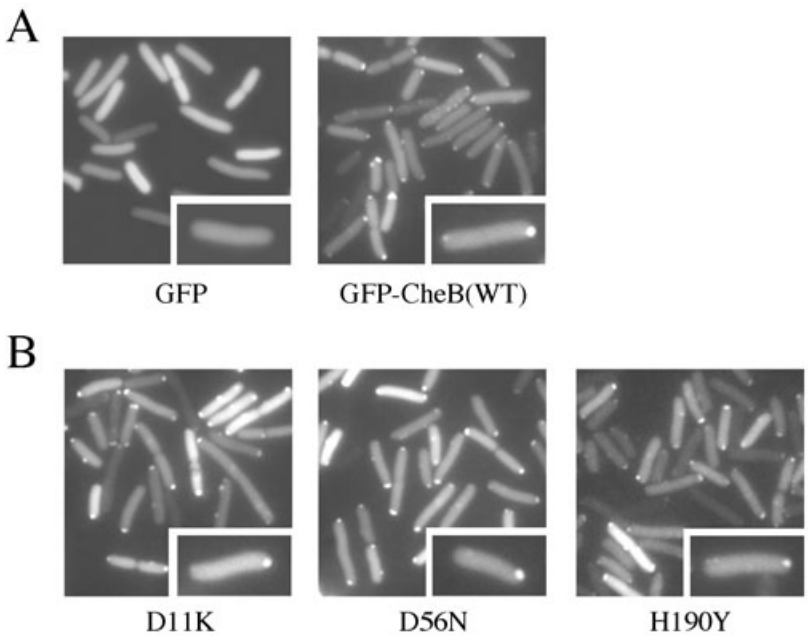

Fig. 4. Subcellular localization of the wild-type and mutant versions of GFP–CheB. Each GFP–CheB fusion protein was expressed in RP4953 (ΔCheB) in the presence of 1 mM arabinose.
A. Cells expressing GFP (left, pDS900) or GFP–CheB (right, pDS901).
B. Cells expressing mutant versions of GFP–CheB: D11K (pDS902), D56N (pDS904) and H190Y (pDS905).

tion from the arabinose promoter, GFP–CheB and its derivatives were expressed under the control of the *trc* promoter without induction, resulting in similar localization patterns (data not shown). These results suggest that the phosphorylation and the catalytic activity of CheB are not essential for its localization.

Both N-terminal and C-terminal domains, when fused to GFP with the linker region, can localize to a cell pole

To examine which region of CheB is responsible for the polar localization, we constructed plasmids encoding GFP fused to various regions of CheB: the N-terminal regulatory domain (N), the linker (L) and the C-terminal catalytic domain (C) (Fig. 1B, see *Experimental procedures*). These plasmids were introduced into RP4953 (Δ*cheB*) cells and examined for ability to support chemotaxis. Cells expressing any fusion did not show chemotaxis, but those expressing the LC fusion spread to some extent (Fig. 2). The linker might serve to stabilize the CheB structure or to help the CheB activity, as cells expressing the C fusion did not spread at all in contrast to those expressing the LC fusion. We next examined the expression levels of these proteins by immunoblotting with anti-GFP antibody (Fig. 3). The C domain fusions were less abundant than the N domain fusions. Both the N and the C domain fusions with the L region expressed at higher levels than those without the L region.

We then examined subcellular localization of GFP–CheB derivatives expressed in RP4953 (Δ*cheB*) cells (Fig. 5). Whereas the N, L and C fusions did not show any localization, the NL fusion localized to a cell pole. The LC fusion also showed significant polar localization, albeit to a lesser extent. When expressed at a higher level (from pSB241), polar localization of the LC fusion was more evident [Fig. 5, LC (overexp.)]. The N fusion did not localize when expressed from pSB111 but, when overproduced from pSB211, a small fraction of cells were occasionally found with faint polar fluorescent spots (Table 1). The L and C fusions did not localize even under overproducing conditions (expressed from pSB231 and 251 respectively; Table 1).

The C-terminal NWETF sequence of the chemoreceptor is not required for the localization of GFP-CheB

A previous study in this laboratory demonstrated that the MTase CheR localizes to a cell pole by binding to the C-terminal NWETF sequence of a high-abundance MCP (Shiomi *et al*., 2002). We examined whether the NWETF sequence is also required for the localization of GFP–CheB. HCB436 cells were transformed first with the plasmid encoding the wild-type or truncated (W550Op, i.e. lacking the four residues of the NWETF sequence) version of Tar and then with the H190Y mutant version of GFP–CheB, the NL and LC fusions. All these GFP–CheB fusion proteins localized to a cell pole even in the absence of the NWETF sequence (Table 1). This result indicates that the localization of CheB does not require its interaction with the NWETF sequence of an MCP.

The P2 domain of CheA serves as a target for the localization of CheB

CheB might interact with the methylation helices of MCPs or with CheA to localize to a cell pole. The histidine kinase

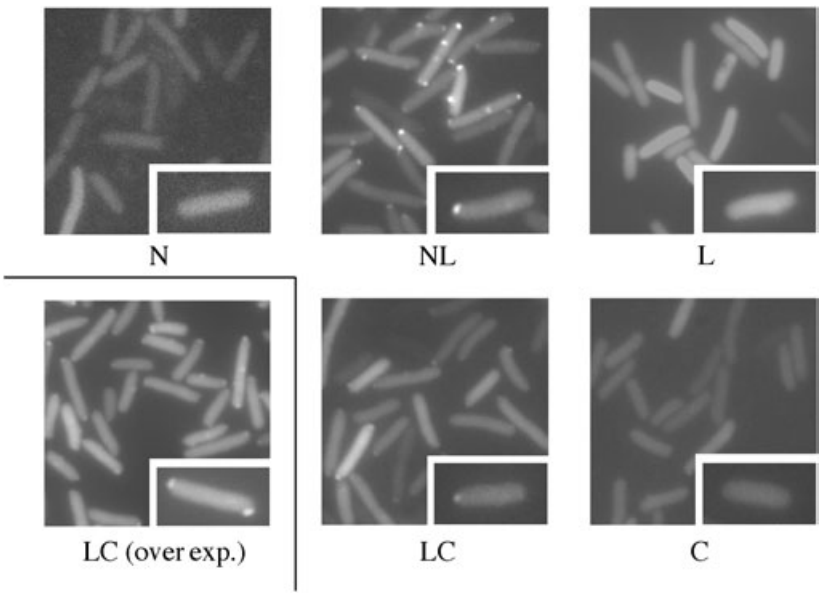

Fig. 5. Subcellular localization of the GFP fusion proteins with the various regions of CheB. Each GFP–CheB fusion protein was expressed in RP4953 (ΔCheB) in the presence of 1 mM arabinose (N, pSB111; NL, pSB121; L, pSB131; LC, pSB141; C, pSB151) or in the absence of IPTG or any other inducer [LC (overexp.), pSB241].

CheA consists of the P1 (phosphotransfer), P2 (binding to CheY and CheB), P3 (dimerization), P4 (kinase) and P5 (coupling to MCPs and CheW) domains (Fig. 6A). First, strains RP437 (wild type for chemotaxis), RP9535 (Δ*cheA*) and UU1121 (*cheA*Δ*P2*) were transformed with the plasmid encoding Tar–GFP (Fig. 6B). This Tar–GFP construct has been shown to localize to cell poles although its localization pattern may not exactly reflect that of wild-type Tar (Homma *et al*., 2004). Immunofluorescence and immunoelectron microscopy show that MCP clusters occupy part of either or both cell poles (Maddock and Shapiro, 1993), whereas Tar–GFP forms large polar foci and is always bipolar. Nevertheless, Tar–GFP is diffused in the absence of CheA and CheW as observed for wild-type Tar in immunofluorescence and immunoelectron microscopy (Maddock and Shapiro, 1993) and, therefore, the Tar–GFP fusion can be used to study essential localization patterns of wild-type Tar in living cells. Tar–GFP localized to poles of cells expressing the mutant CheA protein lacking the P2 domain (UU1121), whereas it localized only partially to poles of cells lacking CheA (RP9535). Secondly, we transformed the same host strains with the plasmid encoding GFP–CheB (Fig. 6B). Unlike Tar–GFP, GFP–CheB did not localize in UU1121 (*cheA*Δ*P2*) cells, whereas GFP–CheR localized normally to a pole (data not shown). These results suggest that the P2 domain of CheA is required for the localization of CheB. Similarly, the deletion of the P2 domain of CheA completely abolished polar localization of the NL fusion (Fig. 7). Interestingly, however, the LC fusion localized to

Table 1. Polar localization of GFP–CheB and its derivatives.

		Host			
			HCB436 (ΔMCPs ΔCheRB)		
Plasmid name	Protein fused to GFP	RP4953 (ΔCheB)	Tar	Tar-W550Op	No receptor
pDS900	None	–	–	–	–
pDS221	CheR	ND	+	–	–
pDS901	CheB	+	+	+	–
pDS902	CheB-D11K	+	+	+	–
pDS904	CheB-D56N	+	+	+	–
pDS906	CheB-H190Y	+	+	+	–
pSB111	CheB (N)	±	ND	ND	–
pSB121	CheB (NL)	+	+	+	–
pSB131	CheB (L)	–	ND	ND	–
pSB141	CheB (LC)	+	+	+	–
pSB151	CheB (C)	–	ND	ND	–

Wild-type, mutant or truncated versions of GFP–CheB were expressed with 1 mM arabinose alone in RP4953 cells lacking CheB or together with the full-length or truncated (W550Op) versions of Tar in HCB436 cells lacking all MCPs, CheR and CheB. At least 200 cells were scored in each observation, which was repeated at least three times. +, >10%; ±, 0–10%; –, no detectable polar localization; ND, not determined.

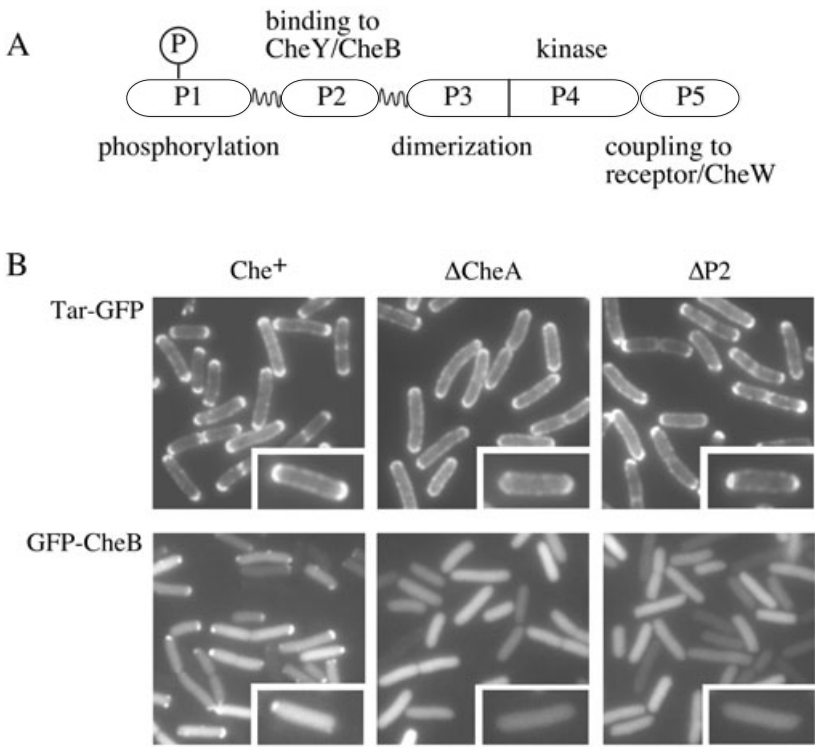

Fig. 6. Effects of *cheA* deletions on the polar localization of CheB.
A. The domain organization of CheA (Morrison and Parkinson, 1997). The domains containing the phosphorylation site (P1), the CheY/CheB-binding domain (P2), the catalytic domain and the receptor/CheW-coupling domain are discrete structural and functional domains.
B. Subcellular localization of Tar–GFP (upper, pDS1020 without an inducer) and GFP–CheB (lower, pDS901 with 1 mM arabinose) in *cheA* deletion strains. The fusion proteins were expressed in RP437 (Che$^+$), RP9535 (ΔCheA) or UU1121 (ΔP2) cells.

a cell pole in the strain lacking the P2 domain. Even in the Δ*cheA* strain, polar localization of the LC fusion was detected in a minor population, suggesting that the LC fusion interacts directly with MCPs that show partial polar localization in the absence of CheA. The localization patterns of the LC fusions in the Δ*cheA* and *cheA*Δ*P2* strains are reminiscent of those of GFP–CheR. These results suggest that the catalytic domain does not contribute to the polar localization of full-length CheB, at least under the conditions tested, but that the C domain, when liberated, can localize to a pole presumably by interacting with MCPs, CheW or the CheA domains other than P2.

To confirm that the NL domain of CheB associates with the P2 domain of CheA, we used a pull-down assay. We fused glutathione *S*-transferase (GST) to the P1 and/or P2 domains of CheA, overexpressed the resulting GST fusion proteins in RP3098 [Δ(*flhA–D*)] cells lacking any MCP or Che protein and added to glutathione column chromatography. Crude cytoplasmic fractions of RP3098 cells expressing GFP–CheB, the NL or the LC derivative

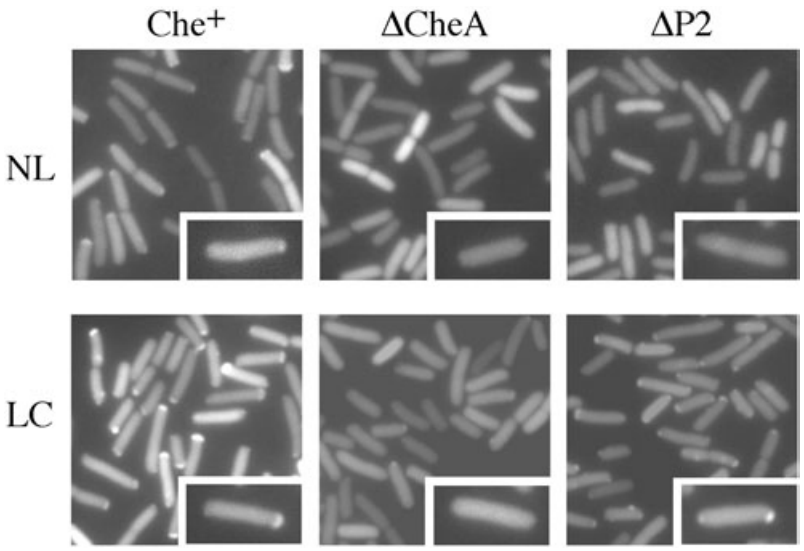

Fig. 7. Subcellular localization of GFP-NL (upper, pSB121) and GFP-LC (lower, pSB141) in *cheA* deletion strains. The fusion proteins were expressed in RP437 (Che$^+$), RP9535 (ΔCheA) or UU1121 (ΔP2) cells in the presence of 1 mM arabinose.

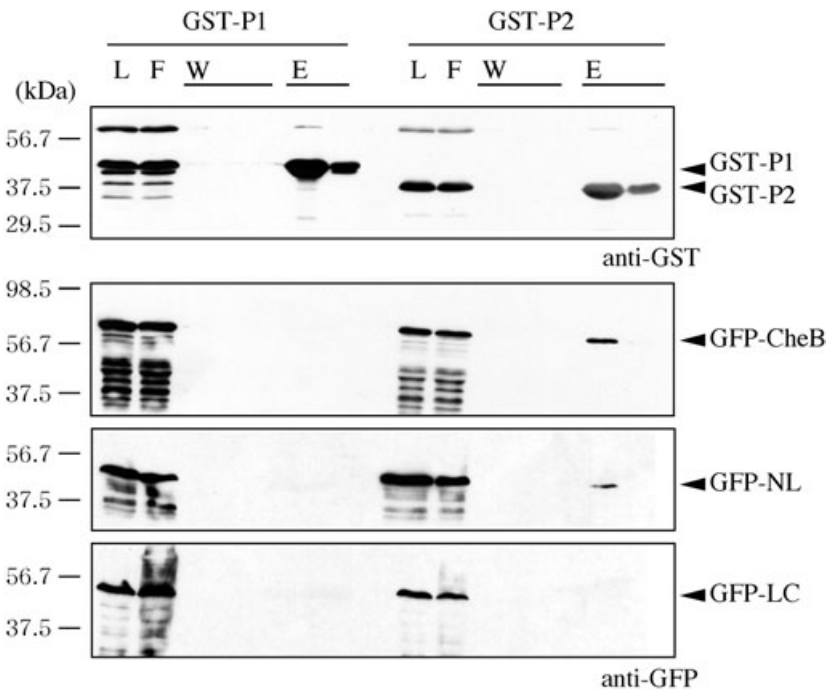

Fig. 8. Binding of CheB to the P2 domain of CheA, determined by GST pull-down assay. First, a cytoplasmic fraction containing GST-P1 (pSB702) or -P2 (pSB707) and then a cytoplasmic fraction containing GFP–CheB (pSB201), NL (pSB221) or LC (pSB241) were applied to a glutathione Sepharose 4B column. The column was washed three times, proteins were eluted twice with glutathione, and all five fractions were applied to SDS-PAGE. GST–CheA and GFP–CheB were detected by immunoblotting with anti-GST and anti-GFP antibody respectively. L, cell lysate; F, flowthrough; W, wash; E, eluate.

were applied to the columns precharged with each GST fusion protein (Fig. 8). When glutathione was applied, GFP–CheB was co-eluted with the GST–P1P2 (not shown) or GST–P2 fusion, but not with GST or the GST–P1 fusion. Similar results were obtained for the NL fusion. In contrast, the LC fusion was not co-eluted with either the GST–P1 or the GST–P2 fusion. It is therefore concluded that only the NL domain is responsible for binding of CheB to the P2 domain of CheA.

Discussion

In this study, we found that the MEase/DAase CheB localizes to a cell pole in an MCP-dependent fashion. The MTase CheR also localizes to a cell pole in the presence of MCP (Shiomi *et al*., 2002). Assembly of such modifying enzymes around their substrates may enhance the efficiencies of receptor methylation and demethylation/deamidation. Barkai *et al*. (2001) and Sourjik and Berg (2002) suggested that CheB is also involved in signal amplification. This process might require polar localization of the protein. The mechanism of polar localization of CheB was found to differ from that of CheR. Binding to the P2 domain of CheA recruits CheB to a cell pole, whereas CheR targets to the C-terminal NWETF sequence of a high-abundance MCP (Fig. 9). CheB interacts with the NWETF sequence but the affinity is rather low ($K_d \approx 160\ \mu M$) (Barnakov *et al*., 2001; 2002). CheB binds to CheA with a K_d value of about 3.2 μM (Li *et al*., 1995), whereas CheR binds to the NWETF pentapeptide with a K_d value of about 2 μM (Wu *et al*., 1996), suggesting that this range of affinity may be required and sufficient for targeting.

Strikingly, the NL and LC domains of the MEase/DAase CheB, when liberated, localized independently to a cell pole. Binding of the NL domain to the P2 domain of CheA is responsible for its polar localization, although that is not the case for the localization of the LC domain. Thus, the localization of full-length CheB seems to depend on the NL domain. It is interesting that the N domain regulates not only the MEase/DAase activity of the C domain but also the localization of the protein. Phosphorylation of the N domain may activate, rather than simply derepress, the C domain as full-length phospho-CheB has a higher MEase/DAase activity than the isolated C domain (Anand *et al*., 1998). However, this phenomenon may not be directly related to the requirement of the N domain for polar localization as the *in vitro* assay did not include CheA. The linker itself might not be involved in the inhibition of the MEase/DAase activity (Anand and Stock, 2002), but its flexibility might help CheB to change its conformation from the closed (inactive) form to the open (active) form (Anand *et al*., 1998). Phosphorylation of the regulatory domain is predicted to shift the equilibrium between the open and closed forms (Anand *et al*., 1998). The fact that the NL and LC fusions, but not the N and C fusions, localize efficiently raises the possibility that the linker might also be involved in stability and/or localization of the protein.

In wild-type cells, the NL fusion showed very modest localization, whereas the LC fusion localized nicely

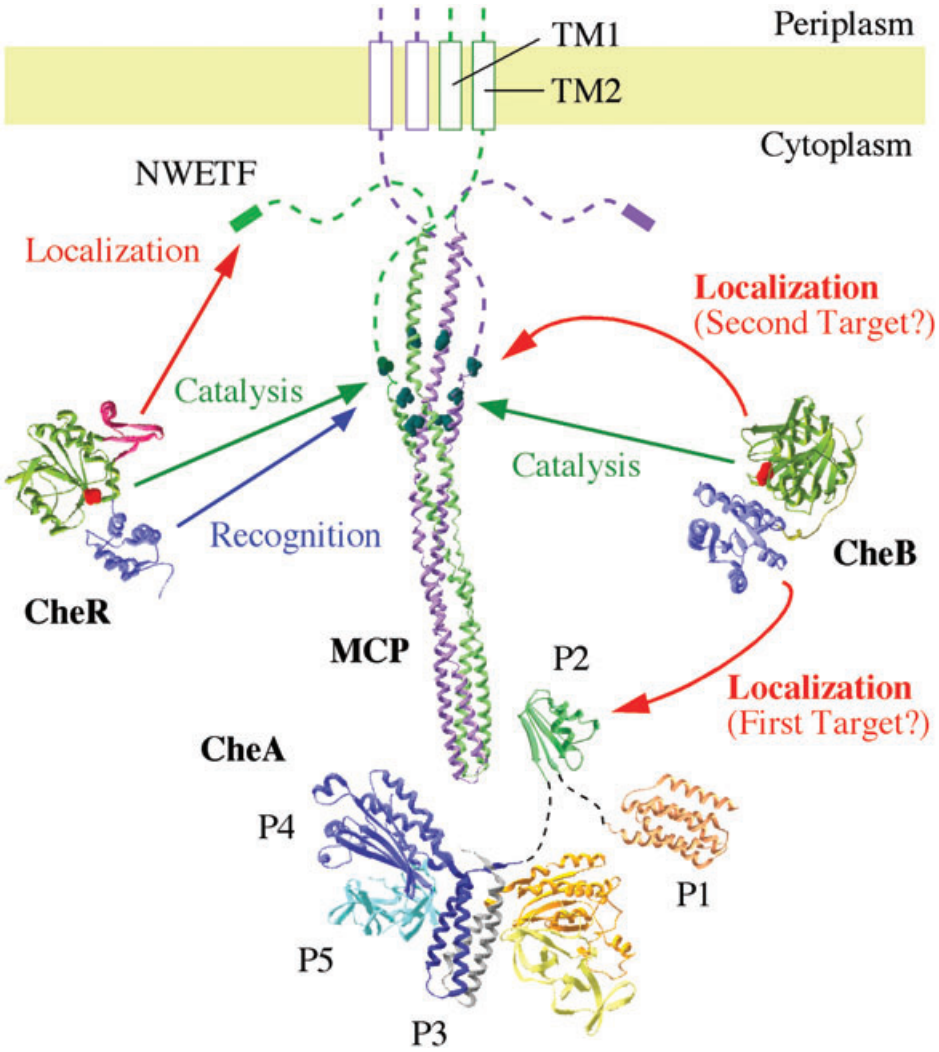

Fig. 9. Mechanisms underlying polar localization of the MEase/DAase CheB and the MTase CheR in an *E. coli* cell. CheB localizes to a cell pole via the binding of its N-terminal regulatory domain to the P2 domain of the histidine kinase CheA, whereas polar localization of CheR requires the binding of its β-subdomain to the C-terminal NWETF sequence of the chemoreceptor (MCP). The C-terminal fragment of CheB (consisting of the linker and the C-terminal catalytic domain) also localizes to a cell pole, presumably by interacting with the methylation helices of MCPs, which might therefore serve as second targets of CheB.

(Fig. 7), a reproducible pattern that seems to be opposite to that in the *cheB* mutant cells (Fig. 5). One likely explanation for this difference is that the LC fusion, but not the NL fusion, competes with wild-type CheB, and so the latter does not localize in its presence. In fact, the NL fusion, even when overproduced, did not show a dominant-negative effect on chemotaxis of wild-type cells (S. Banno and I. Kawagishi, unpublished observation). This interpretation, however, immediately raises further questions, such as why GFP–CheB localizes well in wild-type cells (Fig. 6) and why the LC fusion localizes better in wild-type cells than in *cheB* mutant cells. These are difficult to explain, but one possibility is that the N and C domains might somehow stabilize the interactions of the C and N domains with their targets, respectively, either through a direct N–C interaction or an indirect mechanism, such as an allosteric effect on the receptor–kinase complex. These issues should be addressed experimentally.

Is there any physiological significance of polar localization of the LC fusion? If so, there are two possibilities: (i) a similar fragment might exist *in vivo*; or (ii) the NL and LC domains of full-length CheB might bind to different parts of the receptor–kinase cluster. A C-terminal fragment (≈ 21 kDa) of CheB, which results from a proteolytic cleavage in the linker region, was detected in cell extracts (Simms *et al.*, 1985). We also detected a similar proteolytic fragment of CheB–yellow fluorescent protein (YFP), although the exact cleavage site has not been determined (data not shown). This proteolytic C fragment by itself should be able to target to the receptor–kinase

cluster at a cell pole and might serve to maintain a basal level of MEase/DAase activity or to fine tune signalling, e.g. controlling signal gain. Alternatively, targeting of CheB to the receptor–kinase cluster could be divided into two or more distinct stages (e.g. in a hand-over-hand model discussed below), and the LC domain (as a part of full-length CheB) could be required at a later stage.

In vitro assays demonstrated that CheB is outcompeted by the other response regulator CheY for the binding to the P1–P2 domains of CheA (Li *et al*., 1995). Estimated concentrations and dissociation constants (K_d) of CheA, CheY and CheB predict that most CheA molecules in the cell would be free (i.e. no CheY or CheB is bound). Thus, quick dissociation of CheB from CheA is not necessarily required. However, CheB might be recycled, because the number of CheB molecules is lower than that of MCPs, CheA and CheY. Indeed, phosphorylation of CheB by CheA decreases the affinity for the P2 domain, and CheA inhibits the MEase activity of CheB (Anand and Stock, 2002). This would predict that CheB is not always tethered to the P2 domain of CheA. Rather, the latter domain might serve only as an initial target of CheB.

Possible second target(s) of CheB would be the methylation helices of an MCP that contain substrate sites of the enzyme. This prediction is consistent with the fact that the LC fusion localizes to a pole in the absence of the P2 domain of CheA. In the case of the MTase CheR, the two chemotaxis-specific regions bind to the two distinct regions of MCP: the N-terminal domain and the β-subdomain binding to the methylation helices and the C-terminal NWETF sequence of the chemoreceptor respectively (Shiomi *et al*., 2002). Levin *et al*. (2002) proposed a theoretical model in which CheR moves through the receptor cluster in a hand-over-hand fashion with one of the MCP-binding domains detaching and reattaching to the receptor array before the other domain dissociates. A similar mechanism might operate for the CheB function. It is possible that the C domain of CheB reaches the substrate site on a receptor while the N domain binds to CheA. The distance from the signalling domain of MCP to its methylation sites is about 130 Å, which is about twice as long as the linker (20 residues) of CheB even if it is fully extended. However, the P2 domain might lie closer to the methylation sites in the MCP–CheW–CheA complex. It is also possible that, even after dissociating from the P2 domain, CheB remains hanging around MCPs, being sequestered in a compartment for the adaptation enzymes as postulated by Shimizu *et al*. (2000). In any case, the finding that CheB and CheR target to the different parts of the polar receptor–kinase complex indicates that chemotactic adaptation involves highly ordered arrangement of signalling components.

Experimental procedures

Bacterial strains and plasmids

All strains, plasmids and oligonucleotide primers (for polymerase chain reaction, PCR) used in this study are listed in Tables 2, 3 and 4 respectively. The vector plasmid pTrcHisB (Invitrogen) carries the *trc* promoter, the *lacI*^{q} gene and the *bla* gene. The pTrcHisB-based plasmid pDS1020 encodes the Tar–GFP fusion protein (Homma *et al*., 2004). The vector plasmid pBAD24 (Guzman *et al*., 1995) carries the *araBAD* promoter, the *araC* gene, which encodes the positive and negative regulator of the *araBAD* promoter, and the *bla* gene. The vector plasmid pACYC184 (Chang and Cohen, 1978) carries the *cat* gene. The pACYC184-based plasmid, pLC113 (Ames *et al*., 2002), which carries the wild-type *tar* coding region under the control of the *nahG* promoter, was provided by J. S. Parkinson. Plasmid pEGFP, which encodes the enhanced GFP, and plasmid pGEX-5X-3, which encodes the GST, were purchased from Clontech and Amersham Bioscience respectively.

We constructed a plasmid encoding His_6-GFP. The 0.7 kb *Nhe*I–*Eco*RI fragment of pEGFP was subcloned into the vector pTrcHisB to yield pTrc-His_6-EGFP. The *Nco*I–*Hin*dIII fragments encoding His_6-tagged EGFP from this plasmid were subcloned into the multicloning site of the vector pBAD24 to yield pDS900.

Site-directed mutagenesis of CheB

Site-directed mutagenesis was performed using a two-step PCR method essentially as described previously (Umemura *et al*., 1998). Plasmid pDS901 encoding wild-type *gfp–cheB*

Table 2. Bacterial strains used in this study.

Strain	Genotype	Reference
HCB436	Δ*tsr-7021* Δ(*tar-cheB*) *2234* Δ*trg-100 zbd::*Tn*5 thr leu his met rpsL136*	Wolfe and Berg (1989)
RP3098	Δ(*flhD-flhA*)*4*	Slocum and Parkinson (1983)
RP437	*thi thr leu his met eda rpsL* (wild type for chemotaxis)	Parkinson and Houts (1982)
RP4953	Δ*cheB m62-16 thi his pyrC46 thyA araD139* Δ*lac-U169 nalA rpsL*	J. S. Parkinson (personal communication)
RP9535	Δ*cheA 1643 thi-1 thr-1* (Am) *leuB6 his-4 metF159* (Am) *rpsL1356 ara-14 lacY1 mtl-1 xyl-5 tonA31 tsx-78*	Morrison and Parkinson (1994)
UU1121	Δ*cheA 150-247 thi-1 thr-1* (Am) *his-4 metF159* (Am) *rpsL1356 ara-14 lacY1 mtl-1 xyl-5 tonA31 tsx-78*	J. S. Parkinson (personal communication)

Table 3. Plasmids used in this study.

Plasmid	Protein encoded	Parent	Source
pACYC184	–	P15A	Chang and Cohen (1978)
pBAD24	–	pBR322	Guzman *et al*. (1995)
pDS1015	Tar-W550Op	pLC113	Shiomi *et al*. (2002)
pDS1020	Tar–GFP	pTrcHisB	Homma *et al*. (2004)
pDS900	His_6-GFP	pBAD24	This study
pDS901	His_6-GFP–CheB	pBAD24	This study
pDS902	His_6-GFP–CheB-D11K	pBAD24	This study
pDS904	His_6-GFP–CheB-D56N	pBAD24	This study
pDS906	His_6-GFP–CheB-H190Y	pBAD24	This study
pEGFP	GFP	pBR322	Clontech
pGEX-5X-3	GST	pBR322	Amersham Bioscience
pLC113	Tar	pACYC184	Ames *et al*. (2002)
pSB111	His_6-GFP–CheB (N)	pDS900	This study
pSB121	His_6-GFP–CheB (NL)	pDS900	This study
pSB131	His_6-GFP–CheB (L)	pDS900	This study
pSB141	His_6-GFP–CheB (LC)	pDS900	This study
pSB151	His_6-GFP–CheB (C)	pDS900	This study
pSB201	His_6-GFP–CheB	pTrcHisC	This study
pSB211	His_6-GFP–CheB (N)	pTrcHisC	This study
pSB221	His_6-GFP–CheB (NL)	pTrcHisC	This study
pSB231	His_6-GFP–CheB (L)	pTrcHisC	This study
pSB241	His_6-GFP–CheB (LC)	pTrcHisC	This study
pSB251	His_6-GFP–CheB (C)	pTrcHisC	This study
pSB702	GST–CheA (P1)	pGEX-5X-3	This study
pSB707	GST–CheA (P2)	pGEX-5X-3	This study
pTrcHisB	–	pBR322	Invitrogen
pTrcHisC	–	pBR322	Invitrogen

was used as a template. The primers (Table 4) were synthesized by Invitrogen. The reaction was carried out using Pyrobest DNA polymerase (Takara Shuzo) with 25 cycles of denaturing at 98°C for 10 s, annealing at 57°C for 30 s and extension at 72°C for 1 min.

Construction of the plasmid encoding the GFP–CheB fusion protein

The unique *Hin*dIII site of the coding region of the wild-type *cheB* gene was eliminated by introducing a single base sub-

Table 4. Primers used in this study.

	Primer	
Plasmid	Name	Sequence[a]
pDS900	EGFP-f1(NheI)	GCGCTAGCATGGTGAGCAAGGGC
	EGFP-r1171	CCGCTTACAGACAAGCTGTGACCG
pDS901	CheBf2(BsrGI)	GCTGTACAAGATGAGCAAAATCAGG
	CheBr2(HindIII)	GCAAGCTTTTAAATACGTATCGC
pDS902[b]	CheB-D11K-f	GTTATCTGTCGATAAATCGGCACTGATG
	CheB-D11K-r	CTACAGTGCCGATTTATCGACAGACAAC
pDS904[b]	CheB-D56N-f	GCTGACGCTGAACGTTGAAATGC
	CheB-D56N-r	GCATTTCAACGTTCAGCGTCAGC
pDS906[b]	CheB-H190Y-f	GTTAATTACCCAGTACATGCCGCCC
	CheB-H190Y-r	GGGCGGCATGTACTGGGTAATTAAC
pSB111[c]	CheBN-S134-r	CCCAAGCTTCTAGCTCGCCTTTGCTGC
pSB121[c]	CheBN-L153-r	CCCAAGCTTACAACAACGGCCCCGC
pSB131[d]	S134-CheBC-f	GGGTGTACAAGAGCCTTGCAGCAC
pSB151[e]	L153-CheBC-f	GGGTGTACAAGTTGTTGAGTTCTG
pSB702	CheA-f(BamHI)	CGGGATCCTGGATATAAGCG
	CheA-S167-r(L1)	GGCTCGAGGCGACTGACTGC
pSB707	CheA-R149-f(L1)	CGGGATCCGATTAAGTGTGG
	CheA-G257-r(L2)	GGCTCGAGGCCGGTTGGC

a. Introduced restriction sites are underlined.
b. CheBf2(BsrGI) and CheBr2(HindIII) were also used.
c. CheBf2(BsrGI) was also used.
d. CheBN-L153-r was also used.
e. CheBr2(HindIII) was also used.
f. Plasmid pSB141 was constructed using S134-CheBC-f and CheBr2(HindIII).

stitution without changing the coding amino acid residue. The resulting coding region was amplified by PCR using suitable primers (Table 4) to introduce *Bsr*GI and *Hin*dIII sites at their 5′ and 3′ ends respectively. The *Bsr*GI–*Hin*dIII fragment was cloned between the *Bsr*GI and *Hin*dIII sites of the vector pDS900 to yield the plasmids encoding the wild-type His_6-tagged GFP–CheB fusion protein.

Construction of the plasmids encoding GST fusion proteins with the P1 and/or P2 domain of CheA

The coding regions of the P1 and/or P2 domain of CheA were amplified by PCR using suitable primers (Table 4) to introduce *Xho*I and *Bam*HI sites at their 5′ and 3′ ends respectively. The PCR products were cloned between the unique *Xho*I and *Bam*HI sites of pGEX-5X-3 to yield plasmids encoding GST fused to the P1 and/or P2 domain of CheA.

Detection of GFP–CheB by immunoblotting

RP4953 (ΔCheB) cells expressing GFP–CheB (full length) or any of its derivatives (the N, NL, L, LC and C fusions) were grown at 30°C for 12 h in TG medium [1% tryptone peptone (Difco), 0.5% NaCl, 0.5% (w/v) glycerol] supplemented with ampicillin (Ap). The culture was diluted 1:40 into fresh TG medium supplemented with Ap. Cells were grown with vigorous shaking, harvested at the exponential phase and resuspended in distilled water. The samples were analysed by SDS-PAGE followed by immunoblotting with anti-GFP antibody (Molecular Probes) or with anti-CheB of *Salmonella typhimurium*, which was provided by Dr A. M. Stock.

Observation of subcellular localization of GFP–CheB

Preparation and observation were carried out essentially as described previously (Shiomi *et al*., 2002). RP4953 (ΔCheB) cells expressing GFP–CheB or its derivatives were grown in TG medium with Ap. HCB436 (ΔMCPs ΔCheRB) cells carrying pLC113 encoding the wild-type Tar (QEQE), its derivative pDS1015 encoding the truncated version (W550Op) or the vector pACYC184 were further transformed with the pBAD24-based plasmids encoding GFP–CheB or its derivative. The resulting double transformants were grown in TG medium with Ap and/or Cm supplemented with 1 mM arabinose (for expression of GFP–CheB proteins) and/or 0.5 μM sodium salicylate (for expression of the Tar proteins). Cells were grown with vigorous shaking at 30°C and harvested in late exponential phase. Cells were washed twice with MLM [10 mM potassium phosphate buffer (pH 7.5), 0.1 mM EDTA, 10 mM DL-lactate, 0.1 mM methionine] and resuspended in MLM. Small aliquots of the cell suspensions were spotted on to slide glasses coated with 0.5% agarose and observed under an inverted fluorescence microscope IX70 (Olympus). The images were recorded and processed using a cooled CCD camera CoolNAP-FX/OL (Universal Image Corporation) and the software METAMORPH version 5.0r4 (Roper).

GST pull-down assay for binding of CheB to CheA

A derivative of GST–CheA (P1, P1 + P2 or P2) and a derivative of GFP–CheB (full length, NL or LC) were expressed with 0.5 mM IPTG in the host strain RP3098 [Δ(*flhA–D*)] lacking all Che proteins. The lysate containing each GST–CheA derivative was loaded to a glutathione Sepharose 4B column (Amersham Biosciences), and then the lysate containing a GFP–CheB derivative was applied to the column. The column was washed four times, and the proteins were eluted twice with glutathione. GST–CheA and GFP–CheB proteins were detected by immunoblotting with anti-GST (Sigma) and anti-GFP antibodies respectively.

Acknowledgements

We thank Drs Michael D. Manson and John S. Parkinson for providing plasmids and bacterial strains, Dr Ann M. Stock for antibody, Dr Toshiharu Yakushi for helpful discussion and encouragement, and Dr Howard C. Berg for critical reading of the manuscript. This work was supported in part by grants-in-aid for scientific research from the Japan Society for the Promotion of Science (to D.S. and I.K.).

References

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

Anand, G.S., and Stock, A.M. (2002) Kinetic basis for the stimulatory effect of phosphorylation on the methylesterase activity of CheB. *Biochemistry* **41:** 6752–6760.

Anand, G.S., Goudreau, P.N., and Stock, A.M. (1998) Activation of methylesterase CheB: evidence of a dual role for the regulatory domain. *Biochemistry* **37:** 14038–14047.

Armitage, J.P. (1999) Bacterial tactic responses. *Adv Microb Physiol* **41:** 229–289.

Barkai, N., Alon, U., and Leibler, S. (2001) Robust amplification in adaptive signal transduction networks. *CR Acad Sci Series* **IV** (2)**:** 871–877.

Barnakov, A.N., Barnakova, L.A., and Hazelbauer, G.L. (2001) Location of the receptor interaction site on CheB, the methylesterase response regulator of bacterial chemotaxis. *J Biol Chem* **276:** 32984–32989.

Barnakov, A.N., Barnakova, L.A., and Hazelbauer, G.L. (2002) Allosteric enhancement of adaptational demethylation by a carboxyl-terminal sequence on chemoreceptors. *J Biol Chem* **277:** 42151–42156.

Bornhorst, J.A., and Falke, J.J. (2000) Attractant regulation of the aspartate receptor-kinase complex: limited cooperative interactions between receptors and effects of the receptor modification state. *Biochemistry* **39:** 9486–9493.

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

Chang, A.C., and Cohen, S.N. (1978) Construction and characterization of amplifiable multicopy DNA cloning vehicles derived from the P15A cryptic miniplasmid. *J Bacteriol* **134:** 1141–1156.

Danielson, M.A., Bass, R.B., and Falke, J.J. (1997) Cysteine and disulfide scanning reveals a regulatory alpha-helix in the cytoplasmic domain of the aspartate receptor. *J Biol Chem* **272:** 32878–32888.

Djordjevic, S., and Stock, A.M. (1998) Chemotaxis receptor

recognition by protein methyltransferase CheR. *Nature Struct Biol* **5:** 446–450.

Djordjevic, S., Goudreau, P.N., Xu, Q., Stock, A.M., and West, A.H. (1998) Structural basis for methylesterase CheB regulation by a phosphorylation-activated domain. *Proc Natl Acad Sci USA* **95:** 1381–1386.

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

Falke, J.J., Bass, R.B., Butler, S.L., Chervitz, S.A., and Danielson, M.A. (1997) The two-component signaling pathway of bacterial chemotaxis: a molecular view of signal transduction by receptors, kinases, and adaptation enzymes. *Annu Rev Cell Dev Biol* **13:** 457–512.

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

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

Gestwicki, J.E., Strong, L.E., and Kiessling, L.L. (2000) Tuning chemotactic responses with synthetic multivalent ligands. *Chem Biol* **7:** 583–591.

Guzman, L.M., Belin, D., Carson, M.J., and Beckwith, J. (1995) Tight regulation, modulation, and high-level expression by vectors containing the arabinose P_{BAD} promoter. *J Bacteriol* **177:** 4121–4130.

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

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

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

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

Levin, M.D., Shimizu, T.S., and Bray, D. (2002) Binding and diffusion of CheR molecules within a cluster of membrane receptors. *Biophys J* **82:** 1809–1817.

Li, G., and Weis, R.M. (2000) Covalent modification regulates ligand binding to receptor complexes in the chemosensory system of *Escherichia coli*. *Cell* **100:** 357–365.

Li, J., Swanson, R.V., Simon, M.I., and Weis, R.M. (1995) The response regulators CheB and CheY exhibit competitive binding to the kinase CheA. *Biochemistry* **34:** 14626–14636.

Lybarger, S.R., and Maddock, J.R. (2001) Polarity in action: asymmetric protein localization in bacteria. *J Bacteriol* **183:** 3261–3267.

Maddock, J.R., and Shapiro, L. (1993) Polar location of the chemoreceptor complex in the *Escherichia coli* cell. *Science* **259:** 1717–17123.

Manson, M.D. (1992) Bacterial motility and chemotaxis. *Adv Microb Physiol* **33:** 277–346.

Mizuno, T. (1998) His-Asp phosphotransfer signal transduction. *J Biochem (Tokyo)* **123:** 555–563.

Morrison, T.B., and Parkinson, J.S. (1994) Liberation of an interaction domain from the phosphotransfer region of CheA, a signaling kinase of *Escherichia coli*. *Proc Natl Acad Sci USA* **91:** 5485–5489.

Morrison, T.B., and Parkinson, J.S. (1997) A fragment liberated from the *Escherichia coli* CheA kinase that blocks stimulatory, but not inhibitory, chemoreceptor signaling. *J Bacteriol* **179:** 5543–5550.

Okumura, H., Nishiyama, S., Sasaki, A., Homma, M., and Kawagishi, I. (1998) Chemotactic adaptation is altered by changes in the carboxy-terminal sequence conserved among the major methyl-accepting chemoreceptors. *J Bacteriol* **180:** 1862–1868.

Parkinson, J.S., and Houts, S.E. (1982) Isolation and behavior of *Escherichia coli* deletion mutants lacking chemotaxis functions. *J Bacteriol* **151:** 106–113.

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

Shapiro, L., McAdams, H.H., and Losick, R. (2002) Generating and exploiting polarity in bacteria. *Science* **298:** 1942–1946.

Shimizu, T.S., Le Novere, N., Levin, M.D., Beavil, A.J., Sutton, B.J., and Bray, D. (2000) Molecular model of a lattice of signalling proteins involved in bacterial chemotaxis. *Nature Cell Biol* **2:** 792–796.

Shiomi, D., Okumura, H., Homma, M., and Kawagishi, I. (2000) The aspartate chemoreceptor Tar is effectively methylated by binding to the methyltransferase mainly through hydrophobic interaction. *Mol Microbiol* **36:** 132–140.

Shiomi, D., Zhulin, I.B., Homma, M., and Kawagishi, I. (2002) Dual recognition of the bacterial chemoreceptor by chemotaxis-specific domains of the CheR methyltransferase. *J Biol Chem* **277:** 42325–42333.

Simms, S.A., Keane, M.G., and Stock, J. (1985) Multiple forms of the CheB methylesterase in bacterial chemosensing. *J Biol Chem* **260:** 10161–10168.

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

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

Sourjik, V., and Berg, H.C. (2000) Localization of components of the chemotaxis machinery of *Escherichia coli* using fluorescent protein fusions. *Mol Microbiol* **37:** 740–751.

Sourjik, V., and Berg, H.C. (2002) Binding of the *Escherichia coli* response regulator CheY to its target measured *in vivo* by fluorescence resonance energy transfer. *Proc Natl Acad Sci USA* **99:** 123–127.

Stock, J.B., and Surette, M.G. (1996) Chemotaxis. In Escherichia coli *and* Salmonella typhimurium*: Cellular and Molecular Biology*, 2nd edn. Neidhardt, F.C., Curtiss, R., III, Ingram, J.J., Lin, E.C.C., Low, K.B., Magasanik, B., *et al.*

(eds). Washington, DC: American Society for Microbiology Press, pp. 1103–1129.

Umemura, T., Tatsuno, I., Shibasaki, M., Homma, M., and Kawagishi, I. (1998) Intersubunit interaction between transmembrane helices of the bacterial aspartate chemoreceptor homodimer. *J Biol Chem* **273:** 30110–30115.

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

Wolfe, A.J., and Berg, H.C. (1989) Migration of bacteria in semisolid agar. *Proc Natl Acad Sci USA* **86:** 6973–6977.

Wu, J., Li, J., Li, G., Long, D.G., and Weis, R.M. (1996) The receptor binding site for the methyltransferase of bacterial chemotaxis is distinct from the sites of methylation. *Biochemistry* **35:** 4984–4993.