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Clustering requires modified methyl-accepting sites in low-abundance but not high-abundance chemoreceptors of *Escherichia coli*

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Summary

Chemotaxis signalling complexes of *Escherichia coli*, composed of chemoreceptors, CheA and CheW, form clusters located predominately at cell poles. As the only kind of receptor in a cell, high-abundance receptors are polar and clustered whereas low-abundance chemoreceptors are polar but largely unclustered. We found that clustering was a function of the cytoplasmic, carboxyl-terminal domain and that effective clustering was conferred on low-abundance receptors by addition of the ~20-residue sequence from the carboxyl terminus of either high-abundance receptor. These sequences are different but share a carboxyl-terminal pentapeptide that enhances adaptational covalent modification and allows a physiological balance between modified and unmodified methyl-accepting sites, implying that receptor modification might influence clustering. Thus we investigated directly effects of modification state on chemoreceptor clustering. As the sole receptor type in a cell, low-abundance receptors were clustered only if modified, but high-abundance receptors were clustered independent of extent of modification. This difference could mean that the two receptor types are fundamentally different or that they are poised at different positions in the same conformational equilibrium. Notably, no receptor perturbation we tested altered a predominant location at cell poles, emphasizing a distinction between determinants of clustering and polar localization.

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Introduction

Many bacterial proteins occupy specific sub-cellular niches (Lybarger and Maddock, 2001; Shapiro *et al*., 2002). One well-characterized example is the ternary signalling complex that mediates chemotaxis, which is found in clusters localized in the polar membranes of *Escherichia coli* (Maddock and Shapiro, 1993; Sourjik and Berg, 2000; Shiomi *et al*., 2002; Cantwell *et al*., 2003; Banno *et al*., 2004) and all other species which have been investigated (Alley *et al*., 1992; Maddock and Shapiro, 1993; Harrison *et al*., 1999; Gestwicki *et al*., 2000; Kirby *et al*., 2000). Although the functional consequences of this polar clustering remain largely uncharacterized, clustering and polarity may be inherent features of the signalling systems that mediate bacterial chemotaxis and may be involved in signal amplification and sensory adaptation (Le Moual *et al*., 1997; Li *et al*., 1997; Bray *et al*., 1998; Duke and Bray, 1999; Duke *et al*., 2001; Sourjik and Berg, 2004).

Chemotaxis signalling complexes consist of a core complex of transmembrane chemoreceptors, the histidine kinase CheA, and the coupling protein CheW (Gegner *et al*., 1992; Schuster *et al*., 1993; Liu *et al*., 1997). This complex interacts with response regulator CheY, phosphatase CheZ, methyltransferase CheR, and methylesterase/deamidase CheB, creating a supramolecular complex (see Bren and Eisenbach, 2000; Hazelbauer, 2004). Signalling from receptor to flagellar motor occurs by modulation of CheY phosphorylation that is controlled by autophosphorylation of CheA which, in turn, is controlled by chemoreceptor conformation. An activating conformation is favoured by covalent modification at specific methyl-accepting glutamates in the cytoplasmic domain, whereas an inhibiting conformation is favoured by occupancy at attractant binding sites in the periplasmic domain. The balance between ligand occupancy and modification of methyl-accepting glutamates by the action of CheR and CheB mediates sensory adaptation, and the time lag between the two provides a molecular memory.

Chemoreceptors are homodimers of extended helical bundles that can be divided into a periplasmic ligand-binding domain, a transmembrane domain, and a cytoplasmic signalling and adaptation domain (Falke and Hazelbauer, 2001). Dimers associate at their cytoplasmic, membrane-distal tips to form trimers of dimers (Kim *et al*.,

1999; Studdert and Parkinson, 2004) and trimers are thought to form larger complexes. Such wider interactions are suggested by the extent of cooperativity in signalling (Li and Weis, 2000; Sourjik and Berg, 2004) and by the size of neighbourhoods of adaptational assistance (M. Li and G.L. Hazelbauer, submitted). Interactions that transcend a trimer imply that at least several trimers associate functionally and presumably structurally, and such contacts are likely to occur in receptor clusters (Maddock and Shapiro, 1993; Lybarger and Maddock, 2001).

There are two classes of methyl-accepting chemoreceptors in *E. coli*: the high-abundance receptors, Tsr and Tar, make up ~90% of the cellular complement of receptors, whereas the low-abundance receptors, Trg and Tap, represent only a few per cent of cellular chemoreceptors (Li and Hazelbauer, 2004). Both high-abundance receptors mediate adaptation and efficient chemotaxis in the absence of other receptors (Springer *et al*., 1977). A low-abundance receptor can signal in the absence of other receptors (Hazelbauer and Engström, 1980), but requires the presence of a high-abundance receptor for effective adaptation and chemotaxis (Springer *et al*., 1977; Hazelbauer and Engström, 1980; Feng *et al*., 1997; Weerasuriya *et al*., 1998). High- and low-abundance chemoreceptors are structurally distinct. Low-abundance receptors are almost 20 residues shorter at their carboxyl ends than the high-abundance proteins and lack a pentapeptide sequence, NWETF, found at the extreme carboxyl terminus of high-abundance receptors. The sequence serves as an interaction site for CheR and CheB, substantially enhancing the efficiency of adaptational modification (Wu *et al*., 1996; Djordjevic and Stock, 1998; Barnakov *et al*., 1999; Barnakov *et al*., 2001). The pentapeptide sequence is necessary for efficient adaptational modification of high-abundance receptors (Wu *et al*., 1996; Le Moual *et al*., 1997; Li *et al*., 1997; Barnakov *et al*., 1999) and for their ability to mediate effective chemotaxis (Le Moual *et al*., 1997; Li *et al*., 1997; Feng *et al*., 1999). Furthermore, addition of the final 19 residues of Tsr, which included the pentapeptide, to the carboxyl-terminal end of Trg created a low-abundance receptor that was efficiently modified and mediated effective chemotaxis in the absence of other chemoreceptors (Feng *et al*., 1999), and addition of a comparable segment of Tar to a slightly truncated form of Tap produced intermediate activity (Weerasuriya *et al*., 1998). Taken together, these data argue that the modification-enhancing pentapeptide is crucial in the functional differences between the two receptor classes.

High- and low-abundance chemoreceptors also differ in their ability to form clusters. When present as the sole type of receptor in a cell, all receptors are predominantly polar but only high-abundance receptors are predominantly clustered (Lybarger and Maddock, 2000). In this study, we examined localization of hybrids of the two receptor classes to identify the location of determinants that mediate chemoreceptor clustering. The results of those studies prompted us to investigate more closely the effect of chemoreceptor modification at methyl-accepting sites on clustering and polar localization.

Results

The carboxyl-terminal, cytoplasmic domain carries determinants for efficient clustering

To determine whether the difference in clustering of high- and low-abundance receptors could be correlated with a particular chemoreceptor segment, we produced previously constructed hybrids of a high- and a low-abundance receptor (Fig. 1A) as the only receptor type in cells otherwise wild-type for chemotaxis. The Tsr-Trg hybrids join the amino-terminal domain of Trg to the carboxyl-terminal (cytoplasmic) domain of Tsr (Trsr) or the amino-terminal domain of Tsr to the carboxyl-terminal domain of Trg (Tsrg) (Feng *et al*., 1997; Feng *et al*., 1999); likewise, the Tar-Tap hybrids are fusions between the amino-terminal domain of Tap and the carboxyl-terminal domain of Tar (Tapr) or the amino-terminal domain of Tar and the carboxyl-terminal domain of Tap (Tarp) (Weerasuriya *et al*., 1998) (Table 1). Immunogold microscopy revealed that all hybrid receptors were localized at cell poles. Hybrids with a cytoplasmic domain from a high-abundance receptor (Trsr and Tapr) were efficiently clustered; those with the cytoplasmic domain of a low-abundance receptor (Tsrg and Tarp) were not (Fig. 1B, Table 2, Figs S1, S2 and Table S1). Thus the carboxyl-terminal domains of high-abundance receptors were sufficient to mediate clustering. Hybrids with amino-terminal halves from high-abundance receptors and carboxyl-terminal halves from low-abundance receptors (Tsrg and Tarp) were slightly more clustered than the respective full length low-abundance receptors, indicating that some feature in the amino-terminal half of a high-abundance receptor also promoted clustering.

Efficient clustering is conferred by a short carboxyl-terminal sequence containing the pentapeptide that enhances adaptational modification

A notable difference between the largely conserved carboxyl-terminal domains of high- and low-abundance chemoreceptors is a pentapeptide sequence at the carboxyl terminus of high-abundance receptors. This sequence interacts with CheR and CheB (Wu *et al*., 1996; Djordjevic and Stock, 1998; Barnakov *et al*., 1999), allowing receptors to be modified efficiently by these enzymes and to mediate effective adaptation and thus chemotaxis

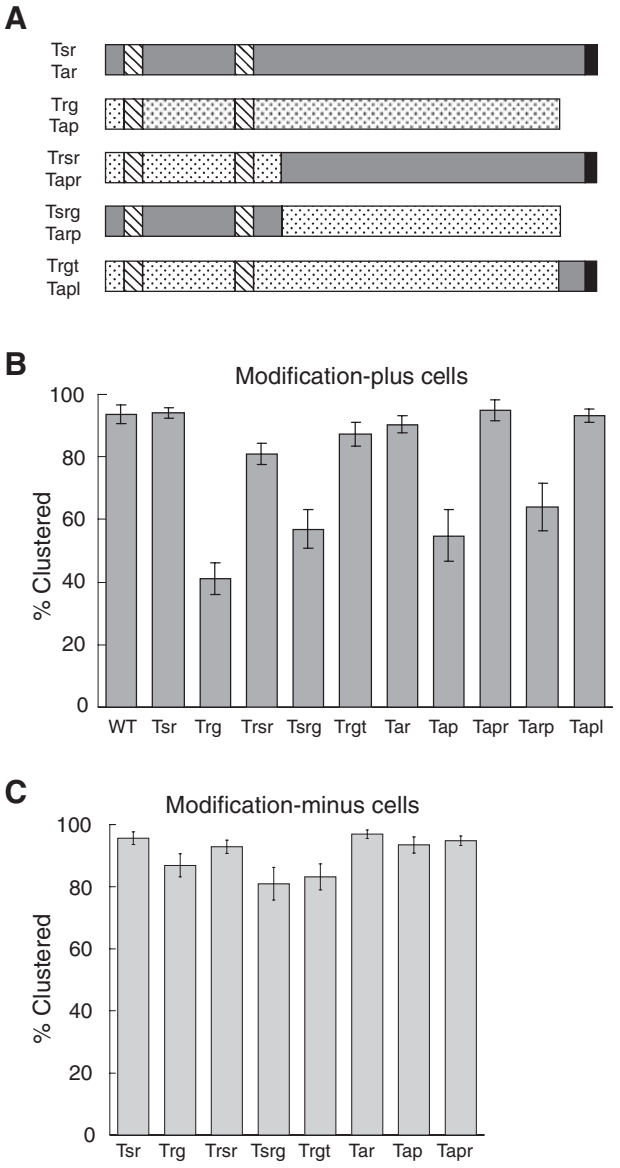

Fig. 1. Organization and clustering of high-abundance, low-abundance and hybrid receptors.
A. Receptor organization. Aligned sequences are symbolized by extended rectangles, shaded grey for sequences from a high-abundance receptor and lightly stippled for those from a low-abundance receptor. Transmembrane domains are hatched and modification-enhancing pentapeptide is black.
B and C. Relative polar clustering of chemoreceptors. Cells possessing 7–10 polar membrane gold particles were analysed. The average per cent clustering (± SEM) is indicated. Analyses were done in: B. cells containing the modification enzymes CheR and CheB (RP437 for WT and UU1250 for all others), or C. cells lacking them (CP553). Clustering percentages for all cell poles with ≥4 gold particles are shown in *Supplementary* Figs S1–S2.

(Weerasuriya *et al*., 1998; Feng *et al*., 1999). To determine whether this difference between the cytoplasmic domains was important for clustering, we determined the localization of hybrids in which the final ~20 residues of a high-abundance receptor were fused to the carboxyl terminus of a low-abundance receptor (Fig. 1A), specifically Trgt, Trg extended by a Tsr carboxyl 'tail' (Feng *et al*., 1999), and Tapl, Tap lengthened by a Tar 'tail' (Weerasuriya *et al*., 1998). The common feature of these two ~20-residue extensions was the modification-enhancing pentapeptide. When produced as the only receptor type in cells otherwise wild-type for chemotaxis, the low-abundance receptors carrying high-abundance tails were localized to the cell poles and exhibited substantially greater clustering than the corresponding low-abundance proteins, which lack the carboxyl extension (Fig. 1B, Table 2, *Supplementary* Figs S1, S2 and Table S1). Thus provision of the modification-enhancing pentapeptide from a high-abundance receptor was sufficient to confer efficient clustering on a low-abundance receptor.

Low-abundance receptors with modified methyl-accepting glutamates are clustered

The ability of the modification-enhancing pentapeptide to mediate clustering of low-abundance receptors implied that adaptational modification influenced localization. Thus we examined receptor localization in cells lacking both modification enzymes. In these cells, all chemoreceptors would have two modified methyl-accepting sites, the two positions at which the respective genes specified glutamines, which are functionally equivalent to methylglutamates. In this situation, all low-abundance as well as high-abundance receptors and receptor hybrids were predominantly at poles and predominantly clustered (Fig. 1C, Table 2, Fig. S3 and Table S1), indicating that low-abundance receptors were clustered if modified.

We then analysed the effect of modification at methyl-accepting sites in a low-abundance chemoreceptor by examining localization of forms of Trg with no (Trg5E), some (Trg2Q) and maximal (Trg5Q) modification in cells lacking chromosomal genes for methyl-accepting receptors and genes for modification enzymes. In these cells, the gene-encoded forms of the receptor would persist. All three Trg forms were found predominantly at poles, but the 5E form was defective in clustering whereas both the 2Q and 5Q forms were extensively clustered (Fig. 2B, Table 2, *Supplementary material*). This showed directly that modification of methyl-accepting sites results in clustering of a low-abundance receptor. As a control, we determined locations of the same forms of Trg in cells containing both the methylesterase and methyltransferase but still missing high-abundance receptors. In such cells, Trg is inefficiently deamidated, methylated and demethylated. However, deamidation is irreversible and over time the cellular complement of Trg loses amides at methyl-accepting sites and cannot compensate by a sufficient level methylation to establish a normal level of modified methyl-accepting glutamates (Park *et al*., 1990; Yama-

Table 1. *E. coli* strains and plasmids.

Strain or plasmid	Description/relevant genotype	Source/reference
RP437	Wild type for chemotaxis	Parkinson and Houts (1982)
KO607	RP437 *Δtsr-7028 Δ(tar-tap)5201 Δtrg-100 ΔrecA56*	Oosawa *et al.* (1988)
UU1250	RP437 *Δtsr-7028 Δ(tar-tap)5201 Δtrg-100 Δaer-1*	Ames *et al.* (2002)
CP553	*Δtsr-7028 Δ(tar-tap-cheR-cheB)2234 Δtrg-100*	Park *et al.* (1990)
pCT1	Plasmid-borne *tsr*	Feng *et al.* (1999)
pGB1	Plasmid-borne *trg*	Burrows *et al.* (1989)
pMK113	Plasmid-borne *tar*	Gardina *et al.* (1992)
pSW2	Plasmid-borne *tap*	Weerasuriya *et al.* (1998)
pHF1	Plasmid-borne *trsr*, coding for a Trg-Tsr hybrid fused at Trg residue 266 with amino-terminal Trg and carboxyl-terminal Tsr	Feng *et al.* (1997)
pHF2	Plasmid-borne *tsrg*, coding for a Tsr-Trg hybrid fused at Trg residue 266 with amino-terminal Tsr and carboxyl-terminal Trg	Feng *et al.* (1997)
pAL75	Plasmid-borne *trgt*, coding for a Trg-Tsr hybrid in which the carboxyl-terminal 19 residues of Tsr are fused to the carboxyl terminus of Trg	Feng *et al.* (1999)
pTarp	Plasmid-borne *tarp*, coding for a Tar-Tap hybrid fused at Tap residue 255 with amino-terminal Tar and carboxyl terminus of Tap	Weerasuriya *et al.* (1998)
pTapr	Plasmid-borne *tapr*, coding for a Tar-Tap hybrid fused at Tap residue 255 with amino-terminal Tap and carboxyl terminus of Tar	Weerasuriya *et al.* (1998)
pTapl	Plasmid-borne *tapl*, coding for a Tap-Tar hybrid in which the carboxyl-terminal 18 residues of Tar are fused to residue 530 of Tap, four residues from the natural carboxyl terminus	Weerasuriya *et al.* (1998)
pAL67	Plasmid-borne *tar6His*, coding for Tar with six histidines at the carboxyl-terminus	This work
pSS1	Plasmid-borne *trg5Q*, coding for Trg with glutamines at all five methyl-accepting sites	Park *et al.* (1990)
pGB10	Plasmid-borne *trg5E*, coding for Trg with glutamates at all five methyl-accepting sites	Park *et al.* (1990)
pJC3	Plasmid-borne *tsr*	Studdert and Parkinson (2004)
pJC34Q	Plasmid-borne *tsr*4Q, coding for Tsr with glutamines at the four major methyl-accepting sites	Studdert and Parkinson (2004)
pJC34E	Plasmid-borne *tsr*4E, coding for Tsr with glutamates at the four major methyl-accepting sites	Studdert and Parkinson (2004)
pNT201	Plasmid-borne *tar*	Borkovich *et al.* (1992)
pAL142	Plasmid-borne *tar*Δpp, coding for Tar deleted of its last five residues, which constitute the modification-enhancing pentapeptide sequence	Barnakov *et al.* (1999)
pAL177	Plasmid-borne *tar4E*, coding for Tar with glutamates at all four methyl-accepting sites	This work
pAL180	Plasmid-borne *tar4Q*, coding for Tar with glutamines at all four methyl-accepting sites	This work

Plasmid-borne receptor genes were under the control of a *tac* promoter.

moto *et al.*, 1990; Feng *et al.*, 1997; Weerasuriya *et al.*, 1998). Thus in the steady state, 5E, 2Q and 5Q forms of Trg will all have low levels of modification at methyl-accepting glutamates. In these conditions, we found that all three receptor forms clustered poorly, although they were still located primarily at a pole (Fig. 2B, Table 2, Table S1 and Fig. S4).

Clustering of high-abundance receptors is not a function of modification at methyl-accepting glutamates

Clustering of low-abundance receptors was influenced by the extent of modification at methyl-accepting sites. To determine whether this was also the case for high-abundance receptors, we examined clustering and polar localization of 4E, 2Q and 4Q forms of high-abundance receptors Tsr and Tar, as well as of a form of Tar (TarΔpp) that lacks the modification-enhancing pentapeptide and therefore functions like a low-abundance receptor (Wu *et al.*, 1996; Le Moual *et al.*, 1997; Li *et al.*, 1997; Barnakov *et al.*, 1999). Each receptor was produced as the only receptor type in a cell containing or lacking the modification enzymes. High-abundance receptors in all modification states and in both cellular environments were polar and clustered (Fig. 3, Table 2, *Supplementary* Table S1). Thus, in contrast to low-abundance receptors, there was no discernible effect of adaptational modification on clustering of high-abundance receptors.

Discussion

This study provides new insight into the determinants of chemoreceptor clustering in *E. coli*. Previously we determined that when expressed alone in cells otherwise wild-type for chemotaxis components, the high-abundance receptors Tsr and Tar clustered at the poles, whereas low-abundance receptors Trg and Tap were polar but hardly clustered (Lybarger and Maddock, 2000). Here we have identified the carboxyl-terminal domain as the segment of the chemoreceptor responsible for clustering. Furthermore, effective clustering could be conferred on either low-abundance receptor by adding ~20 carboxyl-terminal residues of a high-abundance receptor to the carboxyl terminus (Fig. 1, Table 2, *Supplementary* Figs S1, S2 and Table S1). We investigated the possibility that the clustering-conferring property of the carboxyl-

Table 2. Distribution of gold particles in strains expressing receptors in presence or absence of CheR and CheB.

Methyl-accepting receptors in cell[a]	Modifying enzymes in cell[b]	Membrane particles/section	% membrane particles at poles	% polar particles in clusters[c]
Tsr, Tar, Trg, Tap	+	8.2	89	94
none	+	0.5	25	0
Tsr	+	7.9	81	94
Trg	+	10.6	89	41
Trsr	+	13.0	82	81
Tsrg	+	10.9	78	57
Trgt	+	5.7	84	87
Tar	+	8.0	76	90
Tap	+	11.0	81	55
Tapr	+	5.6	89	95
Tarp	+	14.5	85	64
Tapl	+	11.8	77	93
none	–	0.4	41	0
Tsr	–	9.5	85	96
Tar6His[d]	–	6.3	86	97
Tap	–	3.8	85	93
Trg	–	8.2	89	87
Trsr	–	6.5	88	93
Tsrg	–	10.1	85	81
Trgt	–	9.6	88	83
Tapr	–	4.9	84	95
Trg	–	8.2	89	87
Trg5Q	–	10.4	81	85
Trg5E	–	11.8	73	43
Trg	+	10.6	89	41
Trg5Q	+	8.0	87	45
Trg5E	+	13.0	76	42
Tar	–	7.3	86	81
Tar4E	–	7.2	90	91
Tar4Q	–	7.2	86	89
TarΔpp	–	7.4	90	81
Tar	+	6.7	92	88
Tar4E	+	6.1	93	81
Tar4Q	+	6.3	91	83
TarΔpp	+	8.1	90	84
Tsr	–	8.9	86	90
Tsr4E	–	8	90	83
Tsr4Q	–	9.4	87	83
Tsr	+	6.8	89	88
Tsr4E	+	7.9	86	82
Tsr4Q	+	8	88	82

a. For the top entry, strain RP437, receptors were produced from wild-type, chromosomal genes. For all other entries, the receptor gene was plasmid-borne and the host strain devoid of chromosomal methyl-accepting chemoreceptor genes. Induction conditions are listed in experimental procedures. Receptors are grouped in sets of related constructs. The most significant comparisons are between members of such sets.
b. The top entry was strain RP437. The other modification-plus strain was UU1250 and the modification-minus strain was CP553.
c. Shown are the per cent of gold particles clustered at cell poles containing 7–10 gold particles. Clustering percentages for all cells examined are in Table S1.
d. Tar6His, a His-tagged version of Tar capable of mediating aspartate chemotaxis, was used because plasmid-encoded native Tar (pMK113, Table 1) in the CP553 background was present at an unphysiologically high level in the absence of induction. Tar6His has an intracellular distribution pattern similar to that of the untagged Tar in UU1250 (compare Figs S2 and S3).

terminal segment was the result of effective adaptational modification mediated by the conserved NWETF pentapeptide at its carboxyl end and found that clustering of low-abundance receptors in cells lacking other receptors required modification at methyl-accepting glutamyl residues (Figs 1C and 2, Table 2, Fig. S4 and Table S1). In striking contrast, varying the level of adaptational modification on high-abundance receptors did not alter clustering (Fig. 3, Table 2, Table S1). These observations define an additional difference between the two classes of receptors. It is notable that in all conditions we investigated both classes of receptors were located predominantly at a cell pole, regardless of the efficiency of clustering.

Determinants of receptor clustering

We identified two features of chemoreceptors that can facilitate clustering: (i) an as-yet-undefined determinant in the carboxyl-terminal half of high-abundance receptors, and (ii) covalent modification (methylation or amidation) of methyl-accepting sites in the low-abundance receptors.

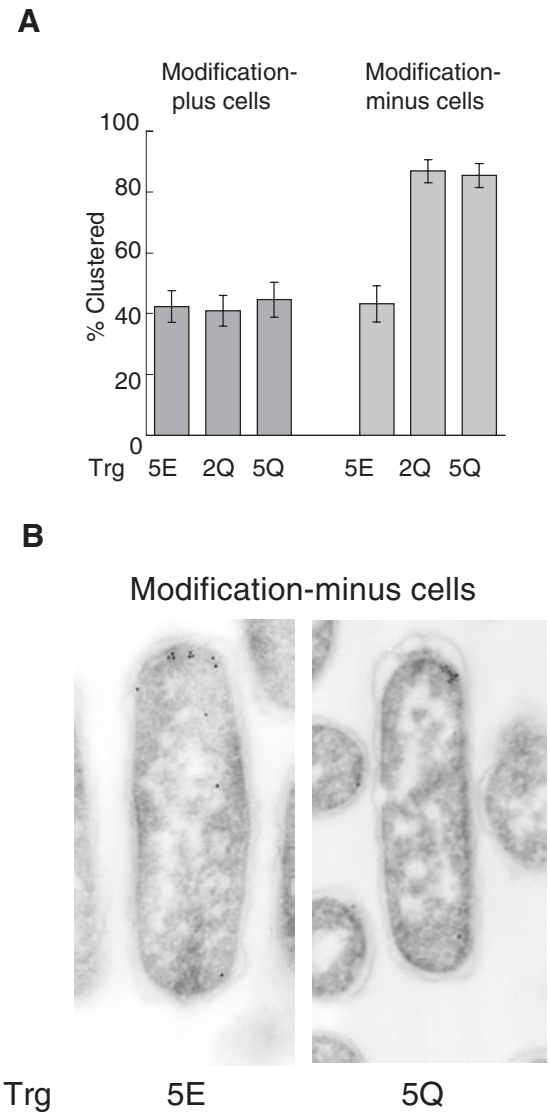

Fig. 2. Clustering of low-abundance receptor Trg as a function of modification state.
A. Relative polar clustering of chemoreceptors. Bar graphs as in Fig. 1 for Trg synthesized with no modifications (5E) at methyl-accepting sites, with two modifications (2Q; wild-type Trg with the two glutamines specified by the wild-type gene) and maximally modified (5Q) in cells lacking or containing the modification enzymes CheR and CheB (modification-minus cells = CP553; modification-plus cells = UU1250). Clustering percentages for all cell poles with ≥4 gold particles are shown in Fig. S4.
B. Representative micrographs of immunogold distributions in cells (CP553) lacking the modification enzymes. The cell on the left contained the unmodified form of Trg (5E) and the one on the right the maximally modified form (5Q).

Neither element affected polar localization of receptors. These observations are consistent with previous studies of receptor localization that documented clustering in cells containing all receptors but lacking CheR or CheB (Lybarger and Maddock, 1999) but inefficient clustering of low-abundance receptors in cellular conditions that resulted in low steady-state levels of methylation (Lybarger and Maddock, 2000). Furthermore, extremes of modification on high-abundance receptors do not disrupt receptor trimers of dimers (Studdert and Parkinson, 2004) and have only very modest effects on polar localization of a Tar-GFP fusion (D. Shiomi, S. Banno, M. Homma and I. Kawagishi, pers. comm.) or of entire signalling complexes as assayed using fusions of derivatives of green fluorescent protein (GFP) to CheY and CheZ (Liberman *et al*., 2004).

Why does lack of modification at methyl-accepting sites of low-abundance receptors preclude clustering? A convincing explanation must consider that the effect appears to be specific for low-abundance receptors even though high-abundance receptors have essentially the same number and arrangement of methyl-accepting sites and are influenced functionally by adaptational modification in the same manner. Thus it seems unlikely that unmodified low-abundance receptors do not cluster because of electrostatic repulsion of multiple, negatively charged glutamates on opposing faces of adjacent receptor dimers as this should be the case for all receptors. An alternative explanation involves the following ideas: (i) there is an equilibrium between clustering and non-clustering receptor conformations, (ii) the equilibrium is shifted toward the clustering conformation by modification at methyl-accepting sites, (iii) high-abundance receptors are positioned sufficiently far toward the clustering conformation that modification is not necessary to observe clustering in the assays we used and (iv) low-abundance receptors are positioned sufficiently far away from the clustering conformation that modification is necessary to observe cluster-

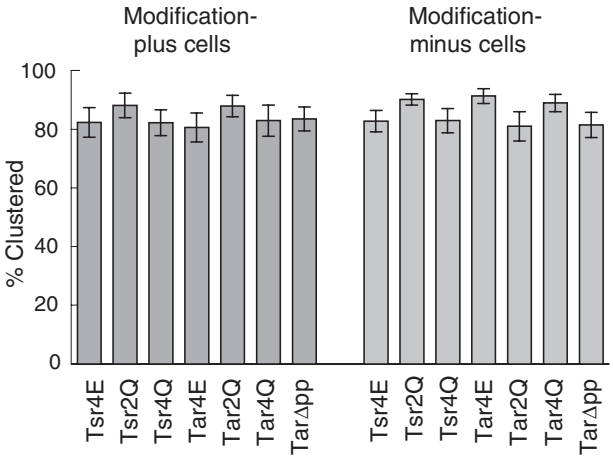

Fig. 3. Clustering of high-abundance receptors as a function of modification state. Bar graphs as in Fig. 1 for Tsr and Tar synthesized with 0, 2 or 4 modifications (glutamines) at methyl-accepting sites, corresponding to Tsr4E or Tar4E, wild-type Tsr or Tar with the two glutamines specified by the wild-type gene, and Tsr4Q or Tar4Q in cells lacking or containing the modification enzymes CheR and CheB (modification-minus cells = CP553; modification-plus cells = UU1250). In addition, we tested TarΔpp, which lacked the carboxyl terminal pentapeptide that enhances adaptational modification.

ing. The conformational equilibrium could be the same one detected by measuring activation and inhibition of receptor-associated kinase, a balance affected by adaptational modification (Borkovich and Simon, 1990; Bornhorst and Falke, 2000; Li and Weis, 2000), and one in which low- and high-abundance receptors with the same extent of modification are at different positions (Barnakov *et al*., 1998).

Clustering of low-abundance receptors in the presence of high-abundance receptors

Direct analysis of the clustering of low-abundance receptors in cells expressing high-abundance receptors has been hampered by technical limitations, but several observations imply that low-abundance receptors *in vivo* participate in clusters composed primarily of high-abundance receptors. Dimers of low-abundance receptors form mixed trimers with high-abundance receptors at physiological levels of expression (Studdert and Parkinson, 2004) and, at these levels of expression, a low-abundance receptor such as Trg is present at ~1/30 the level of high-abundance receptors (Li and Hazelbauer, 2004). Synthetic polyvalent ligands for Trg stabilize large clusters of heterologous chemoreceptors and enhance signal output from the high-abundance receptors in these enforced clusters (Gestwicki and Kiessling, 2002). Thus, it seems likely that low-abundance receptors are distributed more or less uniformly in heterologous trimers and would seldom be part of a receptor trimer of exclusively low-abundance species. In wild-type cells, interaction with high-abundance receptors might drive the conformational equilibrium of the low-abundance receptor towards the clustering conformation. In contrast, high-abundance receptors would not depend on the influence of other receptors, given their inherent propensity for clustering independent of levels of covalent modification. High-abundance receptors may help recruit low-abundance receptors into heterotrimers, effectively incorporating them into clusters regardless of their modification state. This interdigitation of high- and low-abundance receptors in trimers and in higher order clusters would allow adaptational assistance of a receptor lacking the modification-enhancing, carboxyl-terminal pentapeptide by receptors that carry the sequence (Hazelbauer and Engström, 1980; Wu *et al*., 1996; Feng *et al*., 1997; Le Moual *et al*., 1997; Li *et al*., 1997; Feng *et al*., 1999) and may be crucial in effective signalling by receptors that constitute only a few per cent of the cellular complement of chemoreceptors.

Experimental procedures

Bacterial strains, plasmids and growth conditions

Strains and plasmids are described in Table 1. Plasmids pAL177 and pAL180 were constructed from pNT201 by PCR mutagenesis that, respectively, changed codons 295 and 309 to code for glutamate in a single step or changed codon 302 and then codon 491 to code for glutamines in two steps. The fusion constructs between *tap* and *tar* were gifts of Michael D. Manson (Texas A and M University). For immunogold analyses, cells were grown overnight in 1% tryptone plus 0.5% NaCl, supplemented with 100 μg ml^{-1} ampicillin as appropriate, diluted 1:50 in the same medium and grown to OD_{600} 0.5–0.8 before fixation. We adjusted, to the extent possible, receptor expression to approximate total receptor content of a wild-type cell by adding isopropyl β-D-thiogalactoside (IPTG) 60 min before fixation. In the receptor-minus strain UU1250, IPTG concentrations (μM) for the various plasmid-encoded receptor forms were Tsr (0), Tar (10), Tap (0), Trg (50), Trsr (100), Tsrg (0), Trgt (500), Tapr (500), Tarp (0), Tapl (0), Trg5Q (50) and Trg5E (50), and in the receptor-minus, modification-minus strain CP553 were Tsr (0), Tar6His (10), Tap (500), Trg (50), Trsr (50), Tsrg (10), Trgt (250), Tapr (500), Tarp (0), Tapl (0), Trg5Q (50) and Trg5E (50). For experiments in Fig. 3, Tar and its derivatives were induced with 20 μM IPTG, and Tsr and its derivatives with 15 μM IPTG.

Immunoelectron microscopy

Cells were fixed with 3% formaldehyde and 0.1% glutaraldehyde in phosphate buffer (30 mM Na_2PO_4, pH 7.0) for 1–2 h on ice, washed three times with phosphate buffer, incubated 15 min in 1% sodium metaperiodate, washed with phosphate buffer, quenched for 15 min in 50 mM NH_4Cl, washed with water, dehydrated in a graded ethanol series and embedded in LR White resin (Electron Microscopy Sciences) in gelatin capsules at 45–47°C for 2 days. Seventy to ninety nanometre sections were placed on 'sticky' nickel grids (Wright and Rine, 1989).

For immunogold treatments, all solutions were made with double-distilled H_2O and passed through a 0.22 μm filter (ISC BioExpress). We used anti-Tsr serum raised against the highly conserved domain of Tsr (Ames and Parkinson, 1994) that recognizes all four *E. coli* methyl-accepting chemoreceptors. It was diluted 1:500 in PBST (140 mM NaCl, 2 mM KCl, 8 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 0.05% Tween 20) containing 2% BSA and treated for 20 min with acetone powder (Harlow and Lane, 1988) prepared from strain KO607 which lacks methyl-accepting chemoreceptors (Oosawa *et al*., 1988). Grids were immersed in PBST/BSA for 15–30 min in a humidity chamber, incubated in diluted, treated antiserum for 1–2 h, washed three times with PBST, blocked, incubated for 1–2 h in a 1:30 dilution of goat anti-rabbit IgG conjugated to 12 nm of colloidal gold particles (Jackson ImmunoResearch), washed three times with PBST, washed once with double-distilled H_2O and stained with 1% uranyl acetate. Samples were examined with a Philips CM10 electron microscope at 60 or 80 kV.

In each experimental condition we scored the location of all gold particles in a minimum of 160 longitudinal cell sections (Table S1) unless indicated. A cluster was defined as a core group of at least four gold particles within 20 nm, plus all particles within 40 nm of the core. For clarity, figures in the body of this manuscript, as well as the final column in Table 2,

use a subset data from cells containing 7–10 gold particles at a pole. Data for all cell sections examined are found in Table 2, and in the *Supplementary material* in Table S1 and as clustering profiles (Figs S1–S4) that are directly comparable to our previous studies (Lybarger and Maddock, 2000; Skidmore *et al*., 2000).

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Supplementary material

The following material is available from
http://www.blackwellpublishing.com/products/journals/suppmat/mmi/mmi4593/mmi4593sm.htm

Fig. S1. Cluster profiles assessing clustering of hybrids between high-abundance receptor Tsr and low-abundance receptor Trg.

Fig. S2. Cluster profiles assessing clustering of hybrids between high-abundance receptor Tar and low-abundance receptor Tap.

Fig. S3. Cluster profiles assessing clustering of high-abundance receptors Tar and Tsr, and of low-abundance receptors Tap and Trg in the absence of modification enzymes.

Fig. S4. Cluster profiles assessing clustering of low-abundance receptor Trg with different gene-encoded levels of modification at methyl-accepting sites in the absence or presence of modification enzymes.

Table S1. Spatial distribution of chemoreceptors.

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