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Methylation segments are not required for chemotactic signalling by cytoplasmic fragments of Tsr, the methyl-accepting serine chemoreceptor of *Escherichia coli*

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Summary

The serine chemoreceptor Tsr and other methyl-accepting chemotaxis proteins (MCPs) control the swimming behaviour of *Escherichia coli* by generating signals that influence the direction of flagellar rotation. MCPs produce clockwise (CW) signals by stimulating the autophosphorylation activity of CheA, a cytoplasmic histidine kinase, and counter-clockwise signals by inhibiting CheA. CheW couples CheA to chemoreceptor control by promoting formation of MCP/CheW/CheA ternary complexes. To identify MCP structural determinants essential for CheA stimulation, we inserted fragments of the *tsr* coding region into an inducible expression vector and used a swimming contest called 'pseudotaxis' to select for transformant cells carrying CW-signalling plasmids. The shortest active fragment we found, Tsr (350–470), stimulated CheA in a CheW-dependent manner, as full-length Tsr molecules do. It spans a highly conserved 'core' (370–420) that probably specifies the CheA and CheW contact sites and other determinants needed for stimulatory control of CheA. Tsr (350–470) also carries portions of the left and right arms flanking the core, which probably play roles in regulating MCP signalling state. However, this Tsr fragment lacks all of the methylation sites characteristic of MCP molecules, indicating that methylation segments are not essential for generating receptor output signals.

Introduction

Methyl-accepting chemotaxis proteins (MCPs) mediate many chemotactic responses in *Escherichia coli* and other motile bacteria (see Hazelbauer (1992) for review). These transmembrane receptors have a periplasmic sensing domain that monitors chemoeffector levels in the environment through high-affinity binding sites and a cytoplasmic signalling domain that controls the rotational behaviour of the flagellar motors. Favourable and aversive stimuli modulate MCP signal output to elicit appropriate locomotor responses. Attractant increases promote counter-clockwise (CCW) flagellar rotation, which produces smooth, forward swimming. Repellent increases promote clockwise (CW) rotation, which triggers random turns and tumbles. Changes in the MCP methylation state reset signal output, enabling the organism to adapt to current conditions so that it can detect and respond to subsequent stimuli.

Methylation of MCP molecules takes place at several glutamic acid residues located in two segments (called K1 and R1) of the cytoplasmic domain (Kehry and Dahlquist, 1982; Kehry *et al*., 1983b; Terwilliger and Koshland, 1984; Nowlin *et al*., 1987; Rice and Dahlquist, 1991). Two chemotaxis-specific enzymes are involved: CheR, a methyltransferase (Springer and Koshland, 1977), and CheB, a methylesterase (Stock and Koshland, 1978). CheR transfers methyl groups from *S*-adenosylmethionine to MCPs, forming glutamyl methyl esters. CheB hydrolyses MCP methyl groups, regenerating glutamic acid residues and releasing methanol. Mutants lacking CheR or CheB function (Parkinson and Revello, 1978; Goy *et al*., 1977; Yonekawa *et al*., 1983) or with amino acid replacements at the MCP methylation sites (Nowlin *et al*., 1988; Hazelbauer *et al*., 1989; Shapiro *et al*., 1995) can initiate flagellar responses to chemical stimuli but have defects in sensory adaptation. Therefore, changes in MCP methylation state are not needed to generate CCW or CW flagellar signals but rather to regulate the distribution of receptor molecules between these two signalling states. However, it is less clear whether the receptor segments that contain the methylation sites play any role in signal production. K1 and R1 have similar, repetitive primary structures, which are suspected to be recognition motifs for CheR and/or CheB (Terwilliger *et al*., 1986), but which could conceivably represent interaction sites for cytoplasmic signalling

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proteins as well.

The cytoplasmic domains of MCPs form stable ternary complexes with the CheA and CheW signalling proteins to communicate with the flagellar motors through a series of protein phosphorylation reactions (see Parkinson (1993) for review). CheA is a histidine kinase; CheW couples CheA to MCP control. The phosphorelay begins with CheA, which autophosphorylates using ATP. CheA donates its phosphate groups to two aspartate kinases, CheY and CheB, which regulate motor rotation and sensory adaptation, respectively. Phosphorylation of CheY enables it to interact with the flagellar switches to augment CW rotation; phosphorylation of CheB turns on its methylesterase activity. Phospho-CheY and phospho-CheB have high autohydrolysis rates and are very short lived. MCPs regulate the flux of phosphates through this signalling cascade by modulating the autophosphorylation activity of CheA: CW-signalling receptors stimulate CheA; CCW-signalling receptors inhibit CheA.

To explore the mechanisms by which MCPs control CheA activity, we constructed soluble fragments of Tsr, the serine chemoreceptor, that generate CW or CCW signals *in vivo* and stimulate or inhibit CheA *in vitro* (Ames and Parkinson, 1994). Although no longer subject to stimulus control, such fragments evidently carry the chemoreceptor determinants that regulate CheA, including the CheA and CheW contact sites needed to form ternary signalling complexes. The smallest CW-signalling fragment obtained in our initial study lacked the R1 methylation segment but retained the K1 segment. To determine whether the K1 segment is necessary for CheA stimulation, we have constructed derivatives of the original CW-signalling fragment that lack part or all of K1. This report focuses on two K1-less fragments that differ in length by one amino acid residue. The longer one augments CW flagellar rotation *in vivo* and stimulates CheA activity *in vitro*, the other does not. These Tsr fragments cast new light on the structural requirements for MCP signalling activity and its control.

Results

Construction of Tsr fragments

The smallest CW- and CCW-signalling Tsr fragments obtained in our earlier study (Ames and Parkinson, 1994) are shown in Fig. 1. The polypeptide encompassing Tsr residues 290–470, expressed from plasmid pPA56, produces a CheW-dependent CW rotational bias *in vivo* and a CheW-dependent stimulation of CheA autophosphorylation activity *in vitro*. It lacks the R1 methylation segment (Tsr residues 483–507) but has the K1 methylation segment (residues 295–317). A shorter Tsr peptide spanning residues 354–470, expressed from plasmid pPA61, imparts a CCW rotational bias *in vivo*, indicating that the 290–354 segment of Tsr contains information essential for CW signalling. To define more precisely the Tsr region essential for CW signalling activity, we subjected the 290–470 coding fragment to exonuclease digestion and looked for shortened fragments that retained CW function.

The plasmid expression vector used in these experiments, pTM30 (Morrison and Parkinson, 1994), has an IPTG-inducible promoter and efficient translational start just upstream of the cloning site (Fig. 2). Plasmids pPA56 and pPA61 carry *tsr* codons inserted in frame at the *Bam*HI site of pTM30 and produce Tsr polypeptides with three vector-encoded residues at the N-terminus and nine at the C-terminus (Fig. 2). With one important exception, to be described later, all of the shortened derivatives of the 290–470 fragment have these same flanking residues. They were made by opening pPA56 DNA at the *Pst*I site, digesting with *Bal*31 exonuclease for various times and then treating with S1 nuclease to create blunt ends. The shortened *tsr* coding segments were excised by cutting at the downstream *Hin*dIII site and inserted between the *Bam*HI and *Hin*dIII sites in pTM30, yielding constructs with the same flanking sequences as in pPA56.

Pseudotaxis selection for CW-signalling fragments

Motile *E. coli* cells traverse semi-solid agar by swimming through a meshwork of water-filled tunnels. Under conditions that preclude true chemotaxis, the speed of colony

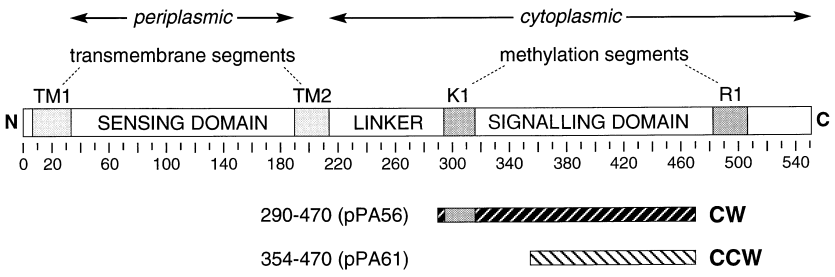

Fig. 1. Domain organization of MCPs. Residue numbers given below the structure are for Tsr, but other *E. coli* MCPs are very similar. Two fragments of the cytoplasmic domain that produce constitutive flagellar signals are shown: plasmid pPA56 encodes a CW-signalling fragment; pPA61 encodes a CCW-signalling fragment.

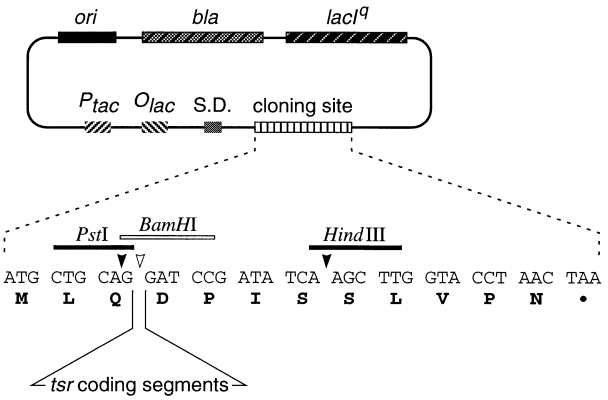

Fig. 2. Key features of the pTM30 cloning/expression vector. Plasmid pTM30, 4363 bp, has the replication origin (*ori*) from pBR322 (Bolivar *et al.*, 1977) and carries a β-lactamase (*bla*) gene conferring resistance to ampicillin. Coding fragments inserted at the cloning site are transcribed from the P_{tac} promoter and translated from an efficient Shine–Dalgarno (S.D.) ribosome-binding site. Insert expression is negatively regulated by Lac repressor (made from the *lacI*^{q} gene) when bound at the O_{lac} control site. The sense strand of the cloning site is shown along with the recognition sequences for restriction enzymes used in this work and their cleavage points (triangles). The vector-encoded amino acids present in Tsr fragment polypeptides are indicated by bold-face, single letter designations.

expansion, a process we call pseudotaxis, depends largely on the cells' pattern of flagellar rotation (Wolfe and Berg, 1989). Cells with a high CCW bias swim forward but seldom turn and consequently spread slowly because they spend much of their time trapped in cul-de-sacs. Cells with a high CW bias also spread slowly because they turn frequently but seldom move ahead. Cells with an intermediate rotational bias, characterized by occasional CW reversals, manoeuvre most rapidly through soft agar because they can swim forward and turn to escape obstructions.

A selection based on pseudotaxis proficiency was devised to identify plasmids that made CW-signalling fragments. Strain RP8691 (*tsr*Δ *cheR*Δ) is generally non-chemotactic and has an extreme CCW rotational bias because its remaining MCPs are unmethylated. Plasmid pPA56, which encodes a CW-signalling Tsr fragment, augmented the colony size of RP8691 on soft agar plates when IPTG was present to induce fragment expression (Fig. 3A). However, pPA61, which encodes a CCW-signalling fragment, had no effect on RP8691 colony size at any IPTG concentration (Fig. 3A). Tethered cell assays confirmed that the pseudotaxis effect of pPA56 was accompanied by a CW shift in flagellar rotation pattern (Fig. 3B). Without induction, over 90% of the pPA56/RP8691 cells rotated exclusively in the CCW direction. At 125 μM IPTG, the optimum concentration for pseudotaxis proficiency, 80% of the cells exhibited varying degrees of CW rotational bias. In contrast, pPA61 did not cause a detectable CW shift in RP8691 flagellar rotation at any inducer level (Fig. 3B).

A reconstruction experiment was done to assess the

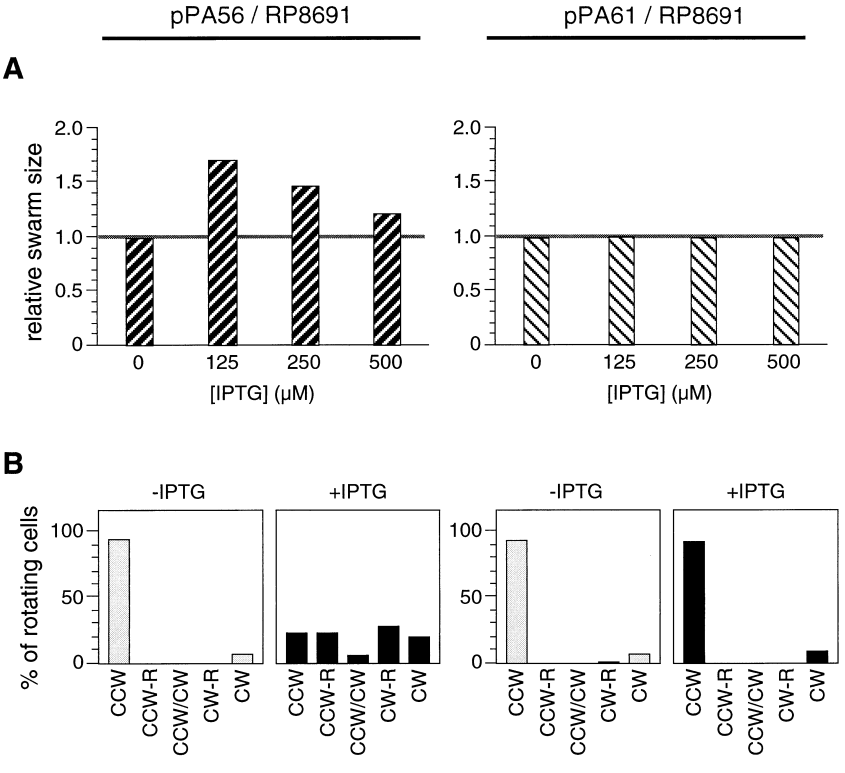

Fig. 3. Behavioural effects of CW- and CCW-signalling *tsr* fragment plasmids in host strain RP8691 (*tsr*Δ *cheR*Δ).
A. Pseudotaxis induction profiles. Semi-solid tryptone agar plates were inoculated with fresh colonies of each strain; colony diameters were measured after incubation at 35°C for 20 h. Sizes were normalized to those on plates without IPTG; values shown are an average of at least five determinations.
B. Flagellar rotation patterns. Strains were grown at 35°C in tryptone broth containing 100 μg ml^{-1} ampicillin. At mid-log phase the cells were harvested, sheared of long flagellar filaments and tethered to microscope slides with flagellar antiserum as described (Parkinson, 1976). Individual rotating cells were observed for 15 s each and classified into one of five rotation patterns (Liu and Parkinson, 1989): exclusively CCW or CW; predominantly CCW or CW, but with occasional reversals (CCW-R and CW-R); and frequently reversing, with no obvious directional preference (CCW/CW). The histograms indicate the percentage of rotating cells assigned to each category. Up to a few per cent of the exclusively CCW or CW cells can be misclassified because of mechanical artifacts in the cell-tethering process (Parkinson, 1976). No attempt was made to correct for the background attributable to such cells. The +IPTG patterns were obtained for cells

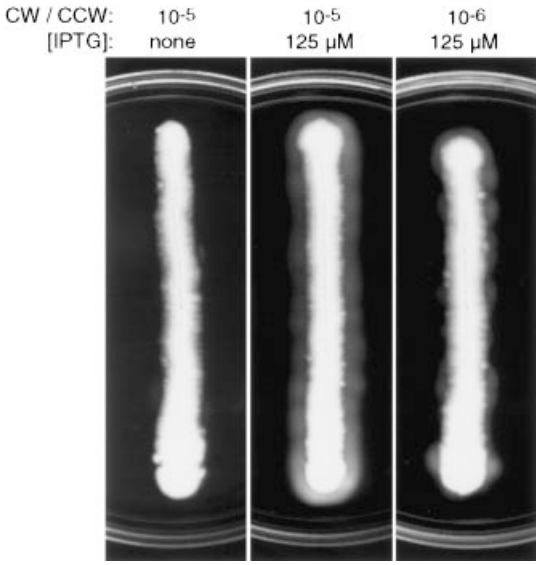

Fig. 4. Reconstruction test demonstrating the efficacy of pseudotaxis selections. Saturated cultures of RP8691 cells carrying pPA56 (CW) or pPA61 (CCW) were mixed at ratios of 10^{-5} and 10^{-6} and streaked on semi-solid tryptone plates with or without IPTG. The plates were incubated for 24 h at 32°C and photographed. The uninduced control shows a few blebs of faster-spreading variants that mostly arise from CW-enhancing mutations in the RP8691 chromosome. Mutations in the CheB methylesterase, rotor switch components or MCPs can produce such compensatory effects (Chen, 1992). The cell streaks on the plates with IPTG show many more blebs, in rough proportion to the number of CW-biased cells in the inoculum.

efficacy of this pseudotaxis system for selecting rare CW-biased cells from a population of CCW-biased ones. RP8691 cells carrying pPA56 (CW) or pPA61 (CCW) were mixed at different ratios and placed on soft agar plates with or without IPTG. Occasional faster-spreading colonies appeared on the plates without IPTG. These represent both pseudorevertants of RP8691, which can arise through CW-enhancing mutations in *cheB* and other chromosomal loci (Chen, 1992), and constitutive mutants of the CW plasmid. Many more pseudotactic blebs appeared on plates with IPTG and in proportion to the frequency of minority CW cells in the reconstructed population (Fig. 4). The detection limit under these pseudotaxis conditions was about 1 CW cell in 10^7 CCW cells.

K1-less fragments with CW-signalling function

Plasmids carrying exonuclease-trimmed *tsr* inserts were transferred to RP8691 to obtain CW-signalling clones by pseudotaxis selection. Transformants were pooled and placed on soft agar plates containing different concentrations of IPTG to permit detection of CW fragments with various signalling strengths. Candidate cells were retested at different IPTG concentrations to determine their pseudotaxis induction profile and their plasmids were extracted to determine the approximate size of their *tsr* insert. From 88 independent isolates, we obtained 78 plasmids with detectably shortened *tsr*

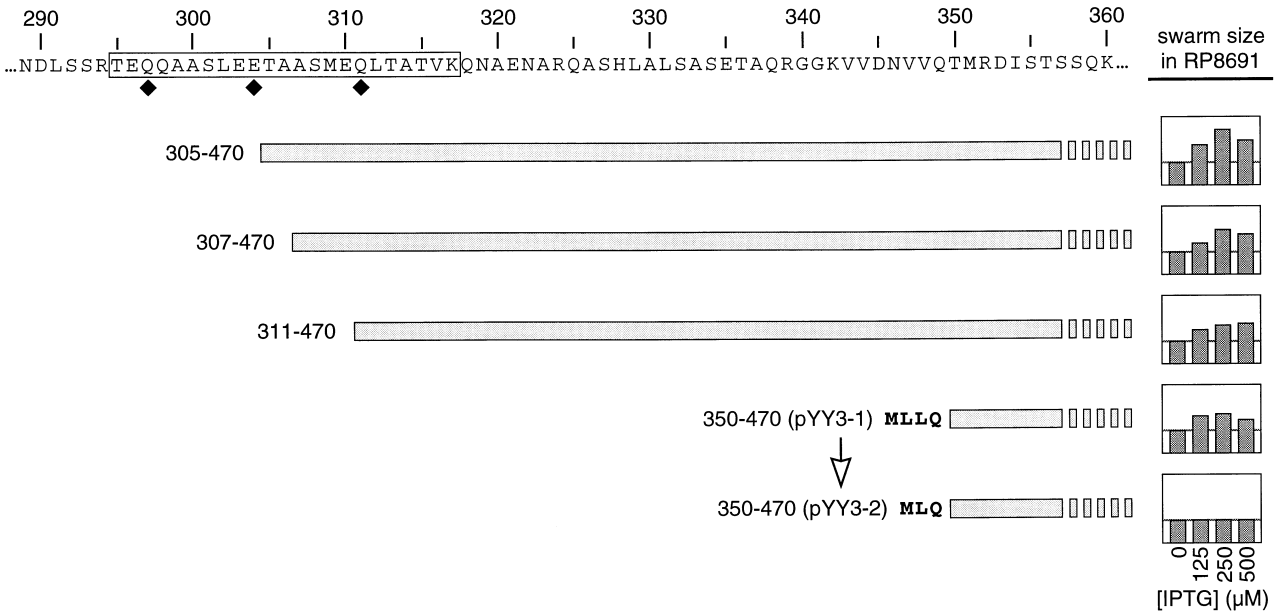

Fig. 5. Truncated Tsr fragments with CW-signalling activity. The Tsr sequence from residues 290 to 360 is shown, with the K1 peptide boxed. Principal methylation sites are indicated by diamonds (Rice and Dahlquist, 1991). Note that two of these residues are glutamines that are deamidatively converted to glutamates by CheB (Kehry *et al.*, 1983a). The shaded bars depict the N-terminal Tsr residues in new CW-signalling fragments obtained from the pPA56 insert, which begins at codon 290. The N-terminal vector-encoded residues in fragments made by pYY3-1 and pYY3-2 are shown in bold. The pseudotaxis induction profiles caused by plasmids expressing each of these fragments were derived and plotted as described in the legend to Fig. 3.

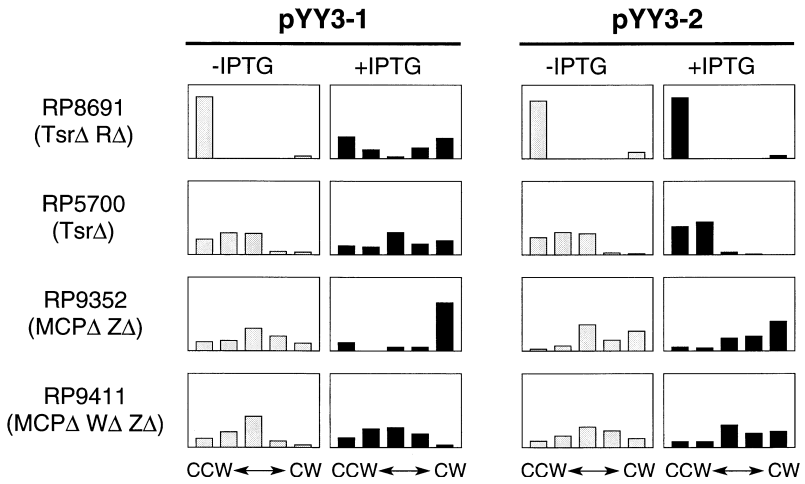

Fig. 6. Epistatic analysis of flagellar rotation effects of pYY3-1 and pYY3-2. Plasmids pYY3-1 and pYY3-2 were transferred into the host strains listed on the left and the rotation patterns of the cells were examined with and without IPTG to induce Tsr fragment expression. The cells were assigned to rotation categories as described in the legend to Fig. 3. Genotypes of host strains are given in Table 1. The critical gene product differences between them are summarized in the figure below the strain designations. Symbols: R (CheR); Z (CheZ); W (CheW); MCP (Tsr + Tar + Tap + Trg).

coding segments. All but one of them appeared to have deletions of 40–75 bp in extent; six of these were chosen for sequence analysis. One plasmid had a considerably smaller insert, corresponding to deletion of about 170 bp, and was also selected for sequencing.

Sequence determinations revealed four different *tsr* inserts in our sample of seven pseudotaxis-selected plasmid clones (Fig. 5). The six largest had end-points within the K1 segment: one started at codon 305, three at codon 307 and two at codon 311. The seventh had an insert that began at codon 350. All four size classes produced similar pseudotaxis profiles, suggesting their Tsr fragments have comparable CW-signalling strengths (Fig. 5). However, they may be somewhat less efficient CW signallers than the 290–470 fragment made by pPA56, whose pseudotaxis profile peaked at a lower IPTG concentration (see Fig. 3).

These findings demonstrate that an intact K1 segment is not essential for CW signalling. Moreover, the shortest insert is just four codons longer than that of pPA61, implying that the CW/CCW boundary lies between Tsr residues 350 and 354. However, the flanking vector sequence in this plasmid (pYY3-1) proved to be slightly aberrant. The leucine codon on the upstream side of the insertion (see Fig. 2) had been duplicated, which should yield a Tsr fragment with a vector-encoded N-terminus of MLLQ, rather than the usual MLQ. Interestingly, the Tsr sequence adjacent to residue 350 is similar — NVVQ (Fig. 5). Could the fortuitous resemblance between the vector-encoded residues of pYY3-1 and the corresponding native sequence in Tsr be responsible for its CW-signalling properties? To find out, we recloned the *tsr* insert of pYY3-1 into a virgin pTM30 vector, producing pYY3-2. Sequence analysis of this new plasmid verified that the duplication had been eliminated. Unlike pYY3-1, pYY3-2 failed to increase the colony size of RP8691 on soft agar at any IPTG concentration, implying that it lacks CW-signalling ability (Fig. 5). As described below, additional behavioural and biochemical tests confirmed major differences in the signalling properties of the Tsr fragments made by these two plasmids.

Signalling properties of the pYY3-1 and pYY3-2 fragments

The flagellar signals generated *in vivo* by pYY3-1 and pYY3-2 were investigated in strains lacking various signalling components (Fig. 6). In RP8691 (*tsr*Δ *cheR*Δ), pYY3-1 caused a CW shift, pYY3-2 did not, a result that corroborates the different pseudotaxis effects of the two plasmids. In RP5700 (*tsr*Δ), a chemotactic strain lacking only the serine chemoreceptor, pYY3-1 caused a CW shift in flagellar rotation, whereas pYY3-2 caused a moderate CCW shift. The fact that both plasmids influence flagellar rotation in RP5700, although in opposite directions, shows that both make Tsr fragments capable of interacting with other components of the flagellar signalling pathway.

In RP9352 (*MCP*Δ *cheZ*Δ), a strain lacking all MCPs, pYY3-1 caused a dramatic CW rotational shift, demonstrating that its signalling activity is not dependent on other chemoreceptors but rather is directed against a downstream component of the signalling pathway, presumably CheA. The inability of pYY3-1 to shift the rotational pattern of RP9411 (*MCP*Δ *cheW*Δ *cheZ*Δ), which lacks CheW in addition to all MCPs, indicates that CheW is essential for CW signalling by pYY3-1. These epistasis tests demonstrated that the signalling behaviour of pYY3-1 was quite similar to the previously described effects of pPA56, the prototypical CW fragment plasmid.

By contrast, the signalling behaviour of pYY3-2 was rather enigmatic. In strains with a nearly full complement

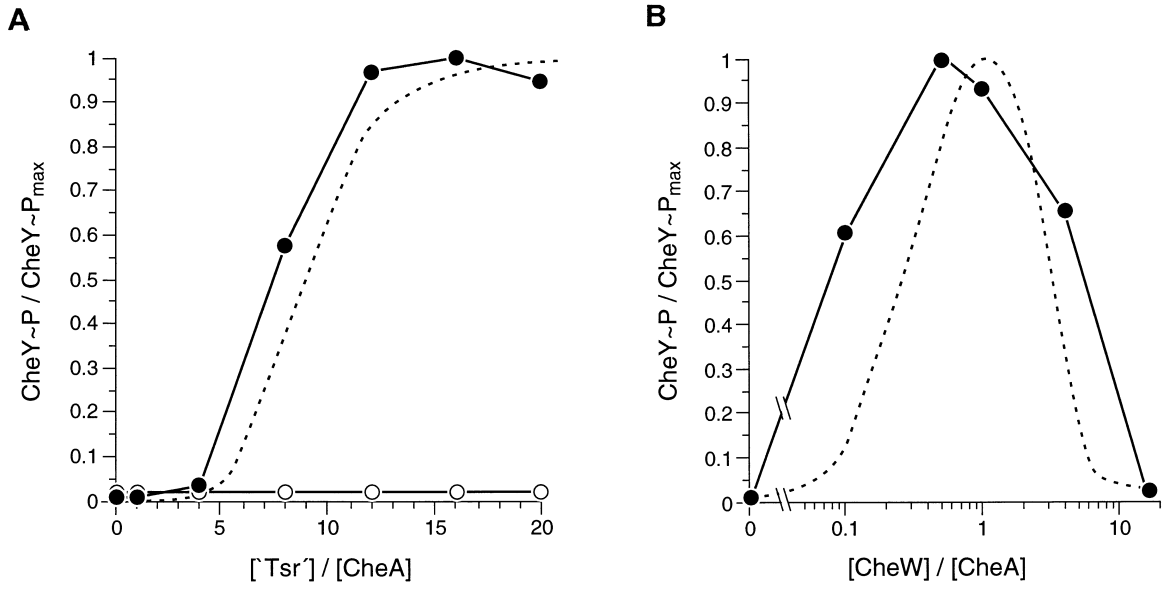

Fig. 7. Stimulation of CheA autophosphorylation by Tsr (350–470) and CheW.
A. Effect of fragment concentration on stimulatory activity. Reactions contained ATP (1 mM), CheA (10 μM), CheW (10 μM), CheY (250 μM) and different amounts of the Tsr (350–470) fragment made by pYY3-1 (●) or pYY3-2 (○). At these stoichiometries, the initial rate of CheY phosphorylation reflects the CheA autophosphorylation rate. CheY phosphorylation values are expressed as a fraction of the maximum rate attained with a 16-fold molar excess of the pYY3-1 fragment to CheA. The dashed line depicts the previously determined behaviour of the Tsr (290–470) fragment made by pPA56 (Ames and Parkinson, 1994).
B. Effect of CheW concentration on stimulatory activity. Reactions contained ATP (1 mM), CheA (10 μM), CheY (250 μM), Tsr (350–470) from pYY3-1 (160 μM) and different amounts of CheW. CheY phosphorylation values are expressed as a fraction of the maximum rate attained at equimolar ratios of CheW to CheA. The dashed line depicts the previously determined CheW dependence of the Tsr (290–470) fragment made by pPA56 (Ames and Parkinson, 1994).

of normal MCPs (RP8691 and RP5700), pYY3-2 showed no capacity to produce CW signals and even a tendency to suppress them. However, in the absence of other MCPs (RP9352), pYY3-2 produced a modest CW rotational shift (Fig. 6). This paradoxical behaviour implies that the pYY3-2 fragment is able to generate CW signals *in vivo* but probably does so much less effectively than the pYY3-1 fragment. Many of its interactions with CheA and CheW molecules might be non-productive but could nevertheless enable it to compete with MCP molecules for these signalling proteins. Thus, in cells containing MCPs, the pYY3-2 fragment might reduce the overall extent of CW signal production (CheA stimulation) by interfering with the formation of functional ternary complexes.

The Tsr fragments made by pYY3-1 and pYY3-2 were purified using the protocol developed previously for the pPA56 fragment (see the *Experimental procedures*) and examined *in vitro* for ability to stimulate CheA autophosphorylation rate, a more direct readout of CW-signalling function. CheA activity was followed in the reactions by measuring the initial rate of CheY phosphorylation, which proceeds through CheA autophosphorylation and subsequent phosphotransfer to CheY. With the assay conditions used (25-fold molar excess of CheY), CheA autophosphorylation was the rate-limiting step in the overall reaction. In assays containing equimolar amounts of CheA and CheW, the pYY3-1 fragment stimulated CheA autophosphorylation as efficiently as the pPA56 fragment, whereas the pYY3-2 fragment produced no detectable stimulation at any concentration tested (Fig. 7A). CheA stimulation by the pYY3-1 fragment seemed to be a co-operative process. No effect was seen at low fragment concentrations, but when fragment levels reached about fivefold excess of CheA, stimulation rose rapidly and saturated at about 15-fold fragment excess. The pPA56 fragment also stimulates CheA co-operatively, but the basis for this behaviour is not yet understood. CheA stimulation by the pYY3-1 fragment was CheW dependent (Fig. 7B) as was its *in vivo* production of CW flagellar signals. Maximal stimulation was attained at roughly equimolar CheW:CheA ratios. Stimulation declined rapidly at CheW concentrations below and above this optimum. Similar behaviour was seen previously with the pPA56 fragment (Ames and Parkinson, 1994). The sharp CheW dependence of the stimulation reaction implies that CheW binds to both CheA and Tsr fragments and serves as a linker in the assembly of ternary receptor complexes (Gegner *et al*., 1992).

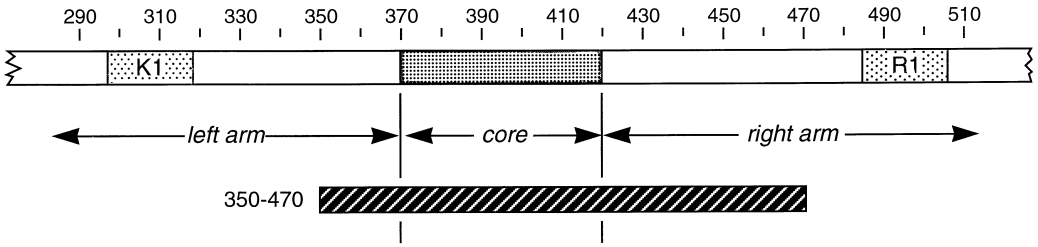

Fig. 8. Features of the Tsr cytoplasmic domain important for CW-signalling activity. A highly conserved 50-residue core may contain the CheA and CheW contact sites needed for ternary complex formation and other determinants essential for CheA stimulation. The CW-signalling 350–470 fragment made by pYY3-1 lacks the K1 and R1 methylation segments, demonstrating that these portions of the arms flanking the core are only involved in controlling, not generating, signal output. The left arm residues around position 350 also play an important, but probably indirect, role in CW signalling. See the text for additional details.

Discussion

MCP signalling states

The signalling properties of MCPs and their cytoplasmic domain fragments are consistent with a two-state model in which receptor molecules have CW- and CCW-enhancing conformations (Ames and Parkinson, 1994). The equilibrium distribution of molecules in each output state determines the overall swimming behaviour of the cell and is subject to modulation by excitatory and adaptive controls. Changes in chemoeffector occupancy shift the CCW/CW proportions to initiate behavioural responses; changes in methylation state restore the pre-stimulus equilibrium to terminate those responses.

CW and CCW signalling involves MCP control of CheA autophosphorylation activity, which in turn regulates the level of phospho-CheY, the effector of CW flagellar rotation. Whereas CCW-signalling receptors inhibit CheA autophosphorylation, CW-signalling receptors stimulate CheA activity several hundredfold over that of uncoupled CheA molecules. Thus, the CW output mode is largely responsible for the amplification of chemoreceptor signals. Although computer simulations suggest a need for additional amplification factors, such as control of the CheY 'phosphatase' CheZ, to account fully for the sensitivity of chemotactic responses (Bray *et al.*, 1993; Hauri and Ross, 1995), there is as yet no direct evidence for other MCP signalling mechanisms.

CheA is coupled to chemoreceptor control by CheW, a mutual binding partner that promotes formation of MCP/CheW/CheA complexes. Once assembled, ternary signalling complexes are long-lived, implying that conformational changes within the complex regulate CheA activity. Both CheW and the receptor signalling domain are essential for stimulatory CheA control, but their mechanistic roles remain unclear. Perhaps through contacts in the ternary complex, CheW and the receptor impose a highly active conformation on CheA. Alternatively, CheW and/or the receptor might contribute catalytic or substrate-binding determinants to the stimulated CheA phosphorylation reaction. CCW-signalling receptors do not require CheW to inhibit CheA, indicating that the MCP output domain probably makes at least one direct contact with CheA in the ternary signalling complex. Whether that binding interaction plays a role in CW signalling is not known.

Generation and control of MCP signals

Single amino acid replacements in many parts of an MCP molecule can 'lock' it in either a CW or CCW output mode, indicating that much of the receptor is dedicated to signal control functions, including stimulus detection, transmembrane signalling and sensory adaptation (Ames and Parkinson, 1988). Control inputs impinge on the signal-generating portion of the receptor, long suspected to be the cytoplasmic region flanked by the K1 and R1 methylation segments (Fig. 8). Indeed, polypeptide fragments from this portion of the serine receptor (Tsr) can elicit CW- or CCW-biased rotational patterns when expressed at high levels in wild-type cells. The smallest CCW fragment so far characterized spans Tsr residues 354–470 (Ames and Parkinson, 1994). In this report, we found that a slightly longer fragment, Tsr (350–470), could generate CW signals. Therefore, both Tsr signals emanate from a region between but not including the K1 and R1 segments. We conclude that the methylation segments are most likely involved only in controlling receptor signal state and not in generating either CW or CCW output.

The CW-signalling properties of the Tsr (350–470) fragment made by plasmid pYY3-1 are comparable to those of native receptors, implying similar mechanisms. The pYY3-1 fragment stimulates CheA autophosphorylation activity in a CheW-dependent manner, so it presumably contains the CheA and CheW contact sites involved in ternary complex formation as well as any other determinants, such as catalytic residues, needed for stimulatory coupling control of CheA. This fragment localizes the CW output machinery of Tsr and, by analogy, other MCPs to a 120 residue tract approximately midway between the K1 and R1 segments (Fig. 8). Within this region lies a highly conserved 'core'

sequence (residues 370–420) common to MCP molecules from widely divergent organisms (Hazelbauer, 1992). The nearly invariant nature of the core implies it is under strong functional selection, consistent with the possibility that it alone specifies the binding sites and other structural features needed for CheA stimulation.

Tsr signalling fragments also carry portions of the 'arms' adjacent to the core (see Fig. 8). All fragments described in this report had the same right arm terminus, so we cannot say what signalling role these residues might play. However, small differences in left arm residues had major effects on fragment signalling activity. For example, the CCW properties of the Tsr (354–470) fragment indicate that left arm residues 350–353 are somehow involved in CW signalling. Similarly, the vector-encoded residues at the N-terminus of Tsr (350–470) fragments had a major influence on their signalling behaviour. The pYY3-1 fragment (N-terminus: MLLQ) had substantial CW activity, whereas the pYY3-2 fragment (N-terminus: MLQ), exhibited little CW activity *in vivo* and failed to stimulate CheA *in vitro*. The flanking residues in the pYY3-1 fragment are similar to those normally adjacent to residue 350 in Tsr (NVVQ) and may be an acceptable substitute for them, implying that residues 346–349 are also involved in CW signalling.

Left arm residues, particularly 346–353, could conceivably play direct roles in CheA stimulation, but their relative lack of sequence conservation suggests they have a more subsidiary function. One possibility is that, like the methylation segments, they participate in regulating transitions between the CW- and CCW-signalling states. A second possibility is that these residues are important to the native structure of the signal-generating core. They might, for example, engender or stabilize secondary structures that extend into the core. Although indirect, functions of this sort could account for our failure to find a broader spectrum of left arm end-points among the CW-signalling Tsr fragments obtained in this study.

Further characterization of Tsr fragments should provide molecular details about the generation and control of MCP signals. Structural comparisons of the CW and CCW output conformations would, for example, clarify the role of arm residues in regulating the signal core. Using pseudotaxis selections analogous to the one in this report, it should also be possible to isolate mutants of the CW-signalling Tsr (350–470) fragment that identify MCP structural determinants, particularly the CheA and CheW interaction sites, that are required for stimulatory control of the CheA kinase.

Table 1. Bacterial strains.

Strain	Relevant genotype	Source/Reference
RP437	Wild type for motility and chemotaxis	Parkinson and Houts (1982)
RP3098	Δ*(flhD–flhA)4*	Smith and Parkinson (1980)
RP5700	Δ*(tsr)7028*	Callahan *et al.* (1987)
RP8691	Δ*(tsr)7028* Δ*(cheR)m58-13*	Chen (1992)
RP9352	Δ*(tsr)7028* Δ*(tar–tap)5201* Δ*(trg)100* Δ*(cheZ)6725*	Liu and Parkinson (1989)
RP9411	Δ*(tsr)7028* Δ*(cheW–tap)2217* Δ*(trg)100* Δ*(cheZ)6725*	Liu and Parkinson (1989)

Experimental procedures

Strains and plasmids

Strains used in this work are listed in Table 1. All were derived from RP437, an *E. coli* K-12 strain that is wild type for chemotaxis (Parkinson and Houts, 1982). All plasmids used or constructed in this work were derivatives of pTM30 (Morrison and Parkinson, 1994) (see Fig. 2). Plasmid pPA56 (CW signalling) carries *tsr* codons 290–470; pPA61 (CCW signalling) carries *tsr* codons 354–470 (Ames and Parkinson, 1994) (see Fig. 1).

DNA manipulations

Restriction enzymes and nucleases were purchased from New England Biolabs and used according to the supplier's recommendations. Unless otherwise specified, treated DNA samples were purified by phenol extraction followed by overnight precipitation at −20°C with ethanol plus ammonium acetate.

Sequence determinations were done by the dideoxy chain termination method, using double-stranded plasmid DNA as template. Oligonucleotide primers complementary to sequences flanking the cloning site in pTM30 were prepared at the Protein–DNA Core Facility, University of Utah Cancer Center.

Construction and cloning of CW-signalling tsr *fragments*

The *tsr* coding segment in pPA56 was shortened from the 5′ end by exonuclease treatment and subsequently released from the plasmid by cutting at the *Hin*dIII site (see Fig. 2). Approximately 30 μg pPA56 DNA was treated with 60 U *Pst*I restriction endonuclease for ~15 h at 37°C. After purification, 25 μg linearized plasmid DNA was mixed with 3 U *Bal*31 exonuclease and incubated at room temperature. Samples containing 5 μg DNA were removed at 10 min intervals and EGTA was added to a final concentration of 200 mM to stop the exonuclease reaction. Approximately four nucleotides per minute per DNA terminus were removed under these conditions. After purification, 5 μg DNA from each time point was treated with 4 U endonuclease S1 for 45 min at room temperature to remove the single-stranded ends left by the exonuclease. After purification, 5 μg samples of DNA

were digested with 40 U *Hin*dIII for ~15 h at 37°C. Restriction fragments containing the truncated *tsr* coding region were recovered from agarose gels with Gene Clean kits (Bio 101).

A linearized pTM30 vector was prepared to clone the truncated *tsr* fragments (see Fig. 2). Approximately 20 μg DNA was successively treated with 60 U *Bam*HI (~15 h at 37°C), 4 U S1 (45 min at room temperature) and 60 U *Hin*dIII (~15 h at 37°C), with repurification of the DNA after each step. Approximately 1 μg samples of *tsr* coding fragments were mixed with ~3 μg linearized vector and treated with 2000 U T4 DNA ligase for ~15 h at 16°C.

To identify inserts with CW-signalling activity, recombinant plasmids were introduced into RP8691 by electroporation and transformants were selected at 37°C on LB agar containing 100 μg ml^{-1} ampicillin. Pools containing about 200 transformant colonies were prepared and streaked on tryptone semisolid agar plates (Parkinson, 1976) containing 50 μg ml^{-1} ampicillin and 125, 250, or 500 μg ml^{-1} IPTG to induce expression of Tsr fragments. After overnight incubation at 35°C, faster-spreading cells were picked from each plate, streaked for single colonies then retested for pseudotaxis proficiency at various IPTG concentrations. Isolates from the same pool with demonstrably different pseudotaxis profiles were regarded as independent. Plasmid DNA was prepared from 96 independent isolates, cut with *Pst*I plus *Hin*dIII and examined by agarose gel electrophoresis to estimate the size of each *tsr* insert. Plasmids from 88 of the 96 isolates had a single insert that fell into one of four general size classes. Compared with the *tsr* insert in pPA56, 10 plasmids had no obvious deletion; 51 were about 30–50 bp shorter; 26 were about 50–80 bp shorter; and one was about 170 bp shorter. Representatives were chosen from the three deleted classes for DNA sequence analysis.

Construction of pYY3-2

Plasmid pYY3-1 contained the shortest *tsr* insert (codons 350–470) yielded by the pseudotaxis selections. However, sequence analysis indicated a duplication of the leucine codon just upstream of the cloning site in the vector (see Fig. 2). To remove the duplication, the pYY3-1 insert was excised by cutting with *Pst*I and *Hin*dIII and recloned into a pTM30 vector, also cut with *Pst*I and *Hin*dIII. DNA sequencing confirmed that the pYY3-1 duplication was not present in pYY3-2.

In vitro *assays of Tsr fragment function*

The Tsr fragments made by plasmids pYY3-1 and pYY3-2 were purified from host strain RP3098 following the procedures developed for the pPA56 fragment (Ames and Parkinson, 1994). The purified polypeptides exhibited identical SDS–PAGE migration rates which were consistent with their predicted molecular mass, so it seems unlikely that they are subject to extensive or differential proteolytic processing *in vivo*. Published procedures were also followed for purification of CheA (Hess *et al*., 1987), CheW (Stock *et al*., 1987), and CheY (Matsumura *et al*., 1984). All proteins were greater than 95% pure by SDS–PAGE analysis and Coomassie brilliant blue staining.

The ability of Tsr fragments to stimulate CheA autophosphorylation activity was measured essentially as described (Ames and Parkinson, 1994). In brief, purified reaction components in TKMD buffer (50 mM Tris pH 8.0, 50 mM KCl, 5 mM $MgCl_2$, 2 mM dithiothreitol) were mixed in various combinations and molar ratios in a total volume of 20 μl and preincubated at room temperature for 60 min. Phosphorylation reactions were initiated by addition of 1 mM [γ-^{32}P]-ATP (~1000 c.p.m. pmol^{-1}) and stopped after 10 s by addition of SDS–PAGE loading buffer. Reactants were separated by electrophoresis on SDS–15% polyacrylamide gels, and the distribution of label was quantified with a Molecular Dynamics PhosphorImager. The concentrations and ratios of components used in the experiments are given in the caption to Fig. 7.

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