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A chemoreceptor from *Pseudomonas putida* forms active signalling complexes in *Escherichia coli*

M. Karina Herrera Seitz, Débora Soto and Claudia A. Studdert

Instituto de Investigaciones Biológicas, Universidad Nacional de Mar del Plata, Mar del Plata, Buenos Aires, Argentina

Correspondence
M. Karina Herrera Seitz
khseitz@mdp.edu.ar

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Chemoreceptors sense environmental stimuli and transmit the information to the flagellar motors via a histidine kinase that controls the phosphorylation level of the effector protein CheY. The cytoplasmic domain of chemoreceptors consists of a long α-helical hairpin that forms, in the dimer, a coiled-coil four-helix bundle. Even though the sequence and general structure of the cytoplasmic domain are strongly conserved within Eubacteria and Archaea, the total length of the α-helical hairpin is variable and defines seven classes of chemoreceptors. In this work we assessed the functional properties of a *Pseudomonas* receptor when assembled in signalling complexes with *Escherichia coli* proteins. Our results show that the foreign receptor does not confer fully chemotactic abilities upon *E. coli* cells, but is able to form active ternary complexes that respond to the specific stimuli by modulating the activity of the associated kinase. The observed responses are subject to adaptation, depending on the presence of the methylation enzymes CheR and/or CheB. The ability of foreign receptors to signal through signalling complexes with non-cognate proteins would allow the use of the well-studied *E. coli* system to reveal the detection specificity of uncharacterized chemoreceptors from other micro-organisms.

INTRODUCTION

The signal transduction pathway that governs chemotaxis is best understood in *E. coli* and is relatively well conserved within Bacteria and Archaea (Wuichet & Zhulin, 2010). Chemical stimuli are sensed by chemoreceptors of different specificities that control the activity of the associated kinase CheA within a ternary complex that also contains the coupling protein CheW. CheA autophosphorylates at a histidine residue and transfers the phosphate group to an aspartate residue in the response regulator CheY. Phosphorylated CheY (CheY-P) interacts with the flagellar switch and causes a change in the rotation of flagella from their default counterclockwise (CCW) direction to clockwise (CW). In *Escherichia coli*, CW rotation of flagella causes tumbles that interrupt the runs driven by CCW-rotating flagella (Hazelbauer & Lai, 2010).

Upon prolonged stimulus exposure, the rapid initial change in CheA activity is followed by a slow recovery of the pre-stimulus activity. Sensory adaptation allows the cells to detect new gradients and is mediated by changes in the methylation level of chemoreceptors, also called MCPs, for methyl-accepting chemotaxis proteins. The methyltransferase CheR adds methyl groups at specific glutamate residues within the cytoplasmic domain of MCPs. CheB is a methyl esterase that hydrolyses these methyl-ester groups. CheB is also a substrate of CheA, and becomes activated by phosphorylation at a specific aspartate residue. Some of the methylatable residues in the receptors are encoded as glutamines and have to be transformed into glutamates by the deamidase activity of CheB (Kehry *et al.*, 1983).

Thus, an attractant stimulus immediately inhibits the kinase and suppresses changes of swimming direction, although the receptors become progressively more methylated by the adaptation machinery and the cells recover their initial frequency of tumbles. Conversely, repellent stimuli activate the kinase, and deamidation/demethylation changes counterbalance this response.

E. coli has four homodimeric MCPs of different detection specificities that possess a ligand-binding periplasmic domain flanked by two transmembrane regions. The cytoplasmic part of these chemoreceptors consists of a HAMP domain followed by an antiparallel α-helical hairpin that forms a four-helix coiled-coil bundle in the MCP dimer. This bundle contains three distinct subdomains: the membrane-proximal methylation/adaptation region, the membrane-distal protein interaction region, and the flexible subdomain between them. The highly conserved protein interaction region, located at the cytoplasmic tip, participates in contacts between dimers to form trimers of dimers (Kim *et al.*, 1999), as well as in contacts with CheW and CheA (Ames & Parkinson, 1994; Park *et al.*, 2006) to form ternary signalling complexes and chemoreceptor clusters.

Abbreviations: CCW, counterclockwise; CW, clockwise; MCP, methyl-accepting chemotaxis protein; TMEA, Tris-(2-maleimidoethyl) amine; YFP, yellow fluorescent protein.

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A comparison of the cytoplasmic domains of more than 2000 MCPs identified symmetrical insertion/deletions of seven-residue stretches (heptads) that defined seven major MCP classes, named according to the number of heptads in the cytoplasmic domain (Alexander & Zhulin, 2007). *E. coli* MCPs belong to the 36H class, meaning that they have 18 heptads in each arm of the cytoplasmic hairpin. Although hexagonal arrangements have been observed in chemoreceptor clusters in different organisms (Briegel *et al.*, 2009, 2012), it is still not clear whether chemoreceptors belonging to different length classes show differences in their higher-order organization, or whether class-specific CheW and/or CheA signalling partners are required for function. Also, it remains to be established whether receptors of different lengths can co-operate within the same cluster.

MCPs are present in variable total numbers and with different predicted topologies in different organisms (Lacal *et al.*, 2010). The presence of many chemoreceptors in some micro-organisms makes it impractical to study them individually through the use of mutants. The well-characterized *E. coli* chemotactic behaviour, and the availability of many different chemotaxis mutants in this organism provide a useful alternative approach to study foreign receptors, provided that they can respond to their specific chemoeffectors when assembled in signalling complexes with *E. coli* proteins. Chemotactic responses to foreign receptors expressed in *E. coli* have been documented in the literature. In one case, a soluble transducer from *Pseudomonas aeruginosa* that belongs to the 36H class has been shown to respond to oxygen (Watts *et al.*, 2011). Another report describes the response towards nitrate mediated by canonical transmembrane transducers from *Shewanella oneidensis* that belong to a length class different from *E. coli* MCPs (Baraquet *et al.*, 2009).

In order to study in detail the functional properties of signalling complexes in which *E. coli* native receptors were replaced by a foreign receptor belonging to a different length class, we chose to clone and express a putative amino acid receptor from *Pseudomonas putida*. The genome of *P. putida* encodes 27 MCPs, 24 of which belong to the 40H class, that is, with 20 heptads in each arm of the cytoplasmic hairpin (Alexander & Zhulin, 2007). Among these are MCPs that are homologous to the previously characterized amino acid receptors from *P. aeruginosa* (Taguchi *et al.*, 1997). In this work, we analysed the signalling abilities of one of these amino acid receptors when expressed in *E. coli* and show that this 40H-class receptor forms functional signalling complexes and modulates CheA kinase activity in response to its cognate ligands.

METHODS

Bacterial strains. *E. coli* strains were derivatives of K12 strain RP437 (Parkinson & Houts, 1982) and carried the following genetic markers relevant for this work: UU1581 [(*flhD-flhB*) 4 Δ(*tsr*)-7028 Δ(*trg*)-100] (Studdert & Parkinson, 2005); UU1250 [Δ(*aer*)1 Δ(*tsr*)7028 Δ(*tar-tap*)5201 Δ(*trg*)100] (Ames *et al.*, 2002); UU1535 [Δ(*aer*)1 Δ(*tsr*)7028 Δ(*tar-cheB*)2234 Δ(*trg*)100 (Bibikov *et al.*, 2004); UU1613 [*tar*-S364C Δ(*tsr*)7028 Δ(*trg*)100 Δ(*tap-cheB*)2234 Δ(*cheA-cheW*)2167 *zec*::Tn*10-2*] (Studdert & Parkinson, 2005).

P. putida KT2440 (Nelson *et al.*, 2002) was used for PCR amplification of the *pctApp* gene (PP_1371) using primers nAP8(F) (5′-CCGACATATGAACAAAAGCCTTCGTTTCAGCC-3′) and nAP9(R) (5′-GATCTGCAGTCAGATGCGGAAGCTGCCC-3′).

Plasmids. Plasmids derived from pACYC184 (Chang & Cohen, 1978) with chloramphenicol resistance were: pKG116 (salicylate-inducible expression vector) (Studdert & Parkinson, 2005), pCS12 (salicylate-inducible wild-type *tsr*) (Studdert & Parkinson, 2005) and pDSo1 (salicylate-inducible wild-type *pctApp*; this work). pDSo1 was built by PCR amplification from *P. putida* KT2440 genomic DNA, cloning into the PCR cloning vector Zero Blunt TOPO (Invitrogen) and subcloning into the salicylate-inducible expression vector pKG116 at the *Nde*I–*Pst*I restriction sites. The entire *pctApp*-coding region was verified by sequencing.

Plasmids derived from pBR322 (Bolivar *et al.*, 1977) with ampicillin resistance were pRR48 (IPTG-inducible expression vector; Studdert & Parkinson, 2005), and pFG1, a pRR48 derivative that expresses a functional yellow fluorescent protein (YFP)–CheZ fusion protein.

Immunoblotting. Cells were pelleted by centrifugation (6000 ***g***) and resuspended at OD_{600} 2 in 10 mM potassium phosphate (pH 7.0) and 0.1 mM EDTA. Cells from 0.5 ml of the suspension were pelleted and lysed by boiling in 50 μl sample buffer (Laemmli, 1970). Lysate proteins were analysed by SDS-PAGE and visualized by immunoblotting with an antiserum directed against the highly conserved portion of the Tsr signalling domain (Ames & Parkinson, 1994). A Cy5-labelled anti-rabbit immunoglobulin (Amersham) was used as secondary antibody. Fluorescence was detected with a Storm 1860 fluorimager (Amersham).

Chemotaxis assays. For assays on semi-solid agar, cells were inoculated into tryptone semi-solid agar plates (Parkinson, 1976) containing 12.5 μg chloramphenicol ml^{-1} and different amounts of sodium salicylate as inducer. Plates were incubated at 30 °C for 7–10 h.

The agarose-in-plug assay was carried out as described elsewhere (Yu & Alam, 1997). In brief, agarose plugs contained 2 % low-melting-point NuSieve agarose (FMC BioProducts), chemotaxis buffer [10 mM potassium phosphate (pH 7.0), 0.1 mM EDTA] and the chemoattractant at a final concentration of 10 mM. Cells were grown at 30 °C to mid-exponential phase in tryptone broth (1 % tryptone, 0.5 % NaCl), harvested by centrifugation at 1000 ***g*** for 5 min, washed twice with chemotaxis buffer, and finally resuspended in the same buffer and introduced into the chamber containing the plugs. Plugs without chemicals were used as negative controls.

Tethered cell assay. UU1250 or UU1535 cells expressing wild-type Tsr or PctApp were grown and pelleted after 2 h of induction. Once washed in 10 mM potassium phosphate (pH 7.0) and 0.1 mM EDTA buffer, cells were resuspended in tethering buffer [10 mM potassium phosphate (pH 7.0), 0.1 mM EDTA, 10 mM sodium lactate, 75 mM NaCl, 0.1 mM L-methionine, 100 μg chloramphenicol ml^{-1}]. These cells were tethered to microscope slides with anti-flagellin antiserum as described by Parkinson (1976), and examined under a phase-contrast microscope, in the absence or presence of amino acid solutions. L-Serine was used as positive control for Tsr. For each strain, at least 100 rotating cells were observed for 15 s each and classified into one of five categories from exclusively CW to exclusively CCW according to their rotational behaviour. Cells were observed within 3 min after the addition of the attractant. The overall percentage of time spent in CW rotation was calculated as a weighted

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sum: the percentage of cells that rotated exclusively CW, plus 0.75 times the percentage of cells rotating predominantly CW, plus 0.5 times the percentage of cells reversing frequently, plus 0.25 times the percentage of cells rotating predominantly, but not exclusively, CCW.

Tris-(2-maleimidoethyl) amine (TMEA) cross-linking assay. Cells were grown at 30 °C to mid-exponential phase in tryptone broth (1 % tryptone, 0.5 % NaCl) containing 25 μg chloramphenicol ml^{-1} and the appropriate concentrations of sodium salicylate, harvested by centrifugation and resuspended at OD_{600} 2 in 10 mM potassium phosphate (pH 7.0), 0.1 mM EDTA. Cell suspensions (0.5 ml) were incubated for 5 min at 30 °C and then treated with 50 μM TMEA (Pierce) for 20 s at 30 °C. Reactions were quenched by the addition of 10 mM *N*-ethylmaleimide. Cells were pelleted and then lysed by boiling in 50 μl sample buffer (Laemmli, 1970). Proteins released from lysed cells were analysed by SDS-PAGE in 10 % acrylamide, 0.05 % bisacrylamide gels and visualized by immunoblotting with Tsr antiserum.

Receptor clustering assay. Receptor clusters were visualized by fluorescence light microscopy in cells expressing a YFP–CheZ fusion protein as a reporter. Cells containing pFG1 (YFP–CheZ under IPTG control) plus the vector control plasmid pKG116 or one of its receptor-coding derivatives were grown at 30 °C in tryptone broth containing 25 μg chloramphenicol ml^{-1}, 100 μg ampicillin ml^{-1}, 100 μM IPTG and 0.45 μM sodium salicylate. Cells were collected at mid-exponential phase and examined essentially as described by Sourjik & Berg (2000). Cell fields were photographed and at least 100 cells were inspected by eye to determine the proportion of individuals with one or more distinct bright spots of fluorescence, which are indicative of a receptor cluster.

RESULTS

Cloning of a putative amino acid chemoreceptor from *P. putida* and its expression in *E. coli*

To identify amino acid receptors from *P. putida*, the protein sequence of the major amino acid chemoreceptor from *P. aeruginosa*, PctA (locus PA4309), was used as query in a BLASTP search against the genome of *P. putida* KT2440 (Nelson *et al.*, 2002). This search revealed an ORF at locus PP_1371, whose predicted protein product, designated PctApp, is homologous to PctA (69 % identity).

The PctApp coding region contains 624 residues (~74 kDa); its product is predicted to have a canonical MCP membrane topology (Fig. 1a). The entire coding sequence for PctApp was amplified by PCR from *P. putida* KT2440 DNA and cloned into an expression vector for *E. coli*, under the control of a sodium salicylate promoter (see Methods).

This vector was introduced into an *E. coli* strain lacking its native receptors, and PctApp expression was induced at different concentrations of sodium salicylate. Total cell lysates were then analysed by SDS-PAGE, Coomassie staining and immunoblotting using an antibody raised against the cytoplasmic domain of the serine chemoreceptor from *E. coli*, Tsr (Fig. 2). In the Coomassie-stained gel, a distinct band appeared as PctApp was induced (Fig. 2a, lanes 2 and 3). This band reacted with anti-Tsr antibody (Fig. 2b, lanes 2–4), confirming that it represents the chemoreceptor PctApp. On the other hand, the expression of plasmid-encoded Tsr in a similar experiment resulted in a strongly immunoreactive band (Fig. 2b, lanes 5–7), even though the same band was barely detectable by Coomassie staining (Fig. 2a, lanes 4–6). Quantification of the intensity of the corresponding bands allowed us to conclude that PctApp reacts with anti-Tsr antibody with a five- to 10-fold reduced efficiency as compared with Tsr.

Functional characterization of PctApp-containing signalling complexes in *E. coli*

An *E. coli* strain lacking all chemoreceptors is motile but nonchemotactic, and displays a predominantly smooth swimming pattern owing to the low level of CheY-P generated by the basal activity of CheA. In such a strain, the expression of any functional chemoreceptor causes an increase in CheY-P levels, due to the activation of CheA in complex with the MCP and CheW. Thus, the cells acquire the ability to tumble and change swimming directions with a certain frequency. If that particular chemoreceptor is able to sense and adapt to specific ligands, when inoculated into semi-solid agar plates that contain a metabolizable attractant the cells might form chemotactic rings as they follow the self-generated attractant gradient.

To determine whether the foreign chemoreceptor PctApp could assemble into functional signalling complexes with *E. coli* proteins, cells expressing PctApp as the only chemoreceptor were subjected to a number of assays, which are described below.

Semi-solid agar plates Cells with no chemoreceptor did not spread in tryptone-containing semi-solid agar plates (Fig. 3a, top-left colony), whereas the expression of Tsr in these cells conferred the ability to form a clearly visible ring upon serine consumption (Fig. 3a, top-right colony). In contrast, PctApp expression did not restore the ability to form a chemotactic ring (Fig. 3a, bottom colonies), even though this chemoreceptor is supposed to be specific for amino acids. This lack of function could be due to the inability of the *P. putida* chemoreceptor PctApp to interact properly with *E. coli* CheW and/or CheA, to defects in kinase control or to problems in methylation-mediated adaptation to stimuli. Thus, additional assays were carried out to elucidate the functional properties of PctApp in this system.

Agarose-in-plug assay In a chamber containing agarose plugs with different substances, chemical gradients form in the solution surrounding the plugs. When a cell suspension is introduced into the chamber, cells respond to their specific attractant by accumulating around the plug that contains it (Yu & Alam, 1997).

This assay was performed with *E. coli* cells expressing no MCP, native Tsr or PctApp, in chambers with plugs containing no attractant, 10 mM L-serine (specific ligand for Tsr and one of the ligands described for PctA; Taguchi *et al.*, 1997) or 10 mM L-aspartate (not a ligand for Tsr or PctA) (Fig. 3b). Tsr-expressing cells clearly accumulated

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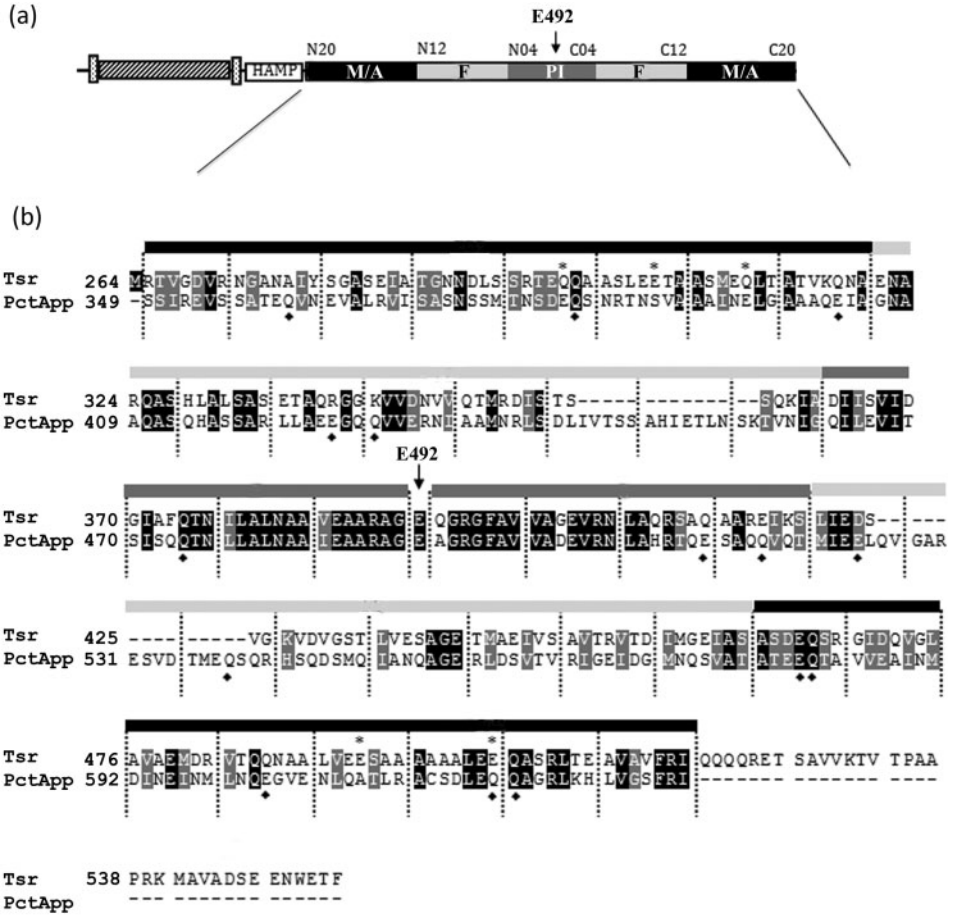

Fig. 1. PctApp sequence analysis. (a) PctApp predicted topology. Stippled vertical bars indicate transmembrane segments. The HAMP domain is followed by the three subdomains of the cytoplasmic hairpin, indicated by shades of grey: M/A, methylation/adaptation region; F, flexible bundle; PI, protein interaction region; E492, residue at the hairpin tip. (b) Alignment between the cytoplasmic domain of PctApp and Tsr. The alignment was made according to Alexander & Zhulin (2007). Vertical dotted lines indicate heptads (fgabcde). The shading of the horizontal bar on top of the alignment indicates the subdomains of the cytoplasmic hairpin as in (a). Black-shaded letters indicate identical residues and grey-shaded letters indicate conservative changes. Asterisks indicate the known methylation sites in Tsr, and rhomboids indicate Glx/Glx residues that occur in PctApp, none of them conforming to the described consensus for methylation sites.

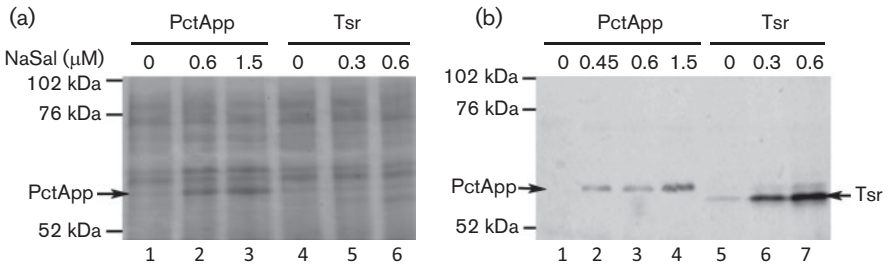

Fig. 2. Expression of PctApp in *E. coli* cells. UU1581 cells (lacking chemotaxis proteins) transformed with pCS12 (Tsr) or pDSo1 (PctApp) were grown to mid-exponential phase under induction conditions with the indicated concentrations of sodium salicylate (NaSal). Total protein extracts were analysed by SDS-PAGE in 10 % polyacrylamide/0.05 % bisacrylamide gels. (a) Coomassie blue G-250 staining. (b) Immunoblotting using an anti-Tsr antibody raised against the cytoplasmic domain of Tsr. The arrows to the left and right indicate the positions of the PctApp and Tsr bands, respectively.

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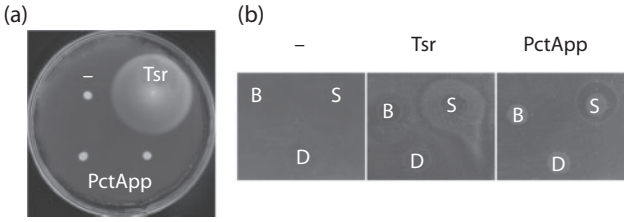

Fig. 3. Chemotaxis assays. UU1250 cells (lacking native receptors) were transformed with empty vector pKG116, pCS12 (Tsr) or pDSo1 (PctApp) and subjected to chemotaxis assays under physiological levels of MCP induction (0.45 μM sodium salicylate). (a) Swimming plates. Semi-solid tryptone plates were inoculated with UU1250 cells expressing no MCP (top left), Tsr (top right) or PctApp (bottom left and right), and incubated at 30 °C for 10 h. (b) Agarose-in-plug assay. UU1250 cells expressing no MCP, Tsr or PctApp were grown to mid-exponential phase, washed, resuspended in phosphate buffer and introduced into a chamber containing agarose plugs with phosphate buffer (B), 10 mM L-serine (S) or 10 mM L-aspartic acid (D). Images were taken 3 min after the cell suspension was introduced into the chamber.

around the serine-containing plug and did not accumulate around the other two control plugs, as expected (Fig. 3b, centre). Notably, PctApp-expressing cells also showed a distinct and strong accumulation around the serine-containing plug (Fig. 3b, right). Even though the appearance of the ring formed by the accumulated cells was quite different from that observed in Tsr-expressing cells, the response was very reproducible and it was ligand-specific, since it was absent in the plugs containing buffer only or L-aspartate. Thus, we conclude that PctApp is able to sense serine as a specific ligand. It was not possible to ascertain at this point whether it was sensed as an attractant or as a repellent.

Kinase control in tethered cells The ability of PctApp to control the CheA kinase from *E. coli* was tested more directly in a flagellar rotation assay. The direction of rotation of cells tethered to a coverslip by a single flagellum was observed and cells were classified into five categories, from exclusively CCW to exclusively CW. In the absence of chemoreceptors, the cells rotated almost exclusively in the CCW direction, reflecting the low levels of CheY-P produced by the basal activity of CheA (Fig. 4a, left panel). When the serine *E. coli* chemoreceptor Tsr was expressed, the cells showed significantly higher levels (26.5 %) of CW rotation (Fig. 4a, middle panel, black bars), indicating CheA activation. The addition of 10 mM L-serine completely suppressed any CW rotation in these cells, as expected for an attractant response (Fig. 4a, middle panel, white bars).

When PctApp was expressed as the sole chemoreceptor in the same strain, the level of CW rotation reached 67.5 %, indicating that PctApp was able to activate *E. coli* CheA at even higher levels than the native receptor Tsr (Fig. 4a, right panel, black bars). This observation confirms the ability of PctApp to assemble into active ternary complexes with the *E. coli* proteins CheW and CheA. Moreover, the addition of 10 mM L-serine caused an immediate inhibition of CheA activity, and the cells rotated exclusively in the CCW direction (Fig. 4a, right panel, white bars), showing that the *Pseudomonas* chemoreceptor is able to control *E. coli* CheA, sensing serine as an attractant.

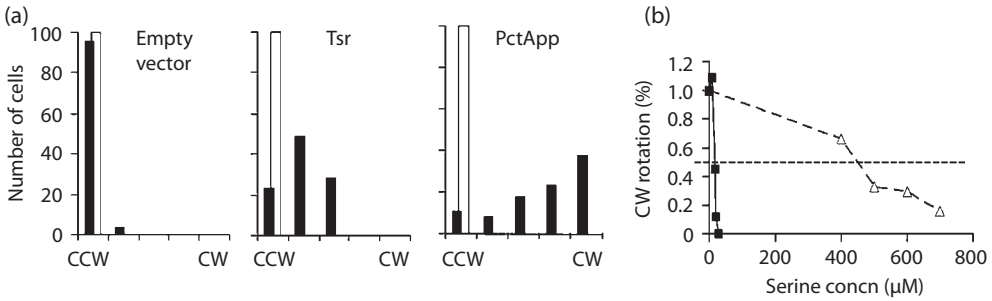

Fig. 4. Kinase control in tethered cells. *E. coli* cells transformed with pKG116 (empty vector), pCS12 (Tsr) or pDSo1 (PctApp) were grown to mid-exponential phase in the presence of 0.6 μM sodium salicylate, washed, and tethered to a coverslip using anti-flagellin (see Methods). One hundred cells were observed for 15 s each, and classified into one of five categories from exclusively CCW to exclusively CW. Results shown are representative of at least three independent experiments. (a) Kinase control in the absence of native receptors. The rotational behaviour of UU1250 cells (lacking native receptors) expressing no MCP (left), Tsr (middle) or PctApp (right) was analysed. Cells were observed in the presence of tethering buffer (black bars) or 10 mM L-serine in tethering buffer (white bars). In the latter case, cells were observed within the first 3 min after addition of attractant. The percentages of CW rotation in the absence of serine (calculated as described in Methods) were: pKG116, 1 %; pCS12, 26.25 %; pDSo1, 67.25 %. After serine addition, the percentage CW was 0 in all cases. (b) PctApp sensitivity. *E. coli* UU1535 cells (lacking native MCPs and CheR/B) were transformed with pCS12 (Tsr, ■) or pDSo1 (PctApp, △). The rotational behaviour was analysed between 0 and 700 μM serine, and results are expressed as a percentage of the initial CW rotation. The initial CW rotation values were 52.5 % for pCS12 and 84 % for pDSo1.

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We used this assay to determine the specificity of PctApp towards L-amino acids (Table 1). All amino acids detected as attractants by PctApp were previously described as attractants for PctA from *P. aeruginosa*.

In order to compare the sensitivities of PctApp and Tsr, we performed the rotation assay in a strain lacking all the MCPs and the methylation enzymes CheR and CheB. The absence of methylation enzymes ensures a homogeneous, unmodified population of receptors. In these cells, both Tsr and PctApp expression led to a high level of CW rotation (52.5 and 84 %, respectively). Rotating cells were exposed to different concentrations of L-serine and the resulting level of CW rotation was calculated (Fig. 4b). Tsr showed an approximate half-response at 17 μM serine, whereas PctApp required a concentration as high as 500 μM. This result indicates that, in the absence of sensory adaptation enzymes, PctApp in *E. coli* cells is about 30-fold less sensitive to serine than is Tsr.

Taken together, the rotation assays allow us to conclude that PctApp is able to activate the CheA kinase and control its activity in response to specific attractants, in complex with *E. coli* proteins.

Table 1. Specificity of the PctApp reponse

E. coli UU1250 cells (lacking native receptors) carrying pDSo1 (PctApp) were induced at 0.45 μM sodium salicylate, and the response to a 10 mM solution of each amino acid in tethering buffer was tested by the tethered cell assay. Results were calculated as (percentage of CW in amino acid solution/percentage of CW in buffer) and expressed as: strong response (+, values of 0–0.25), weak response (+/−, values between 0.25 and 0.75) and no response (−, values >0.75).

Amino acid	Attractant response
Buffer	−
L-Serine	+
Glutamine	−
Methionine	+
Leucine	+/−
Aspartic acid	−
Glycine	+
Tryptophan	+
Asparagine	+/−
Cysteine	+
Isoleucine	−
Phenylalanine	−
Threonine	+
Valine	+/−
Alanine	+
Arginine	−
Glutamic acid	+
Lysine	+
Tyrosine	−
Histidine	−
Proline	+

Methylation-dependent adaptation

To determine whether cells that express PctApp as their only chemoreceptor were able to adapt to a serine stimulus, the rotational behaviour of such cells was analysed at different times after the addition of serine. Cells lacking native *E. coli* receptors and expressing PctApp showed 79.5 % CW rotation and responded to the addition of 700 μM L-serine with a 70 % decrease in CW rotation (Fig. 5, bottom-left panel). After 30 min, the cells recovered significant levels of CW rotation, indicating that they adapted to the stimulus to a certain extent. This behaviour was similar to the one observed when the same strain expressing Tsr was stimulated with 100 μM L-serine (Fig. 5, top-left panel).

Conversely, when the same experiment was carried out in the receptorless strain that also lacks modification enzymes, neither Tsr- nor PctApp-expressing cells showed any indication of adaptation to the serine stimulus (Fig. 5, top- and bottom-right panels), indicating that for both receptors the observed adaptation was dependent on CheR and/or CheB. To assess the effect of *E. coli* methylation enzymes on PctApp under steady-state, unstimulated conditions, the rotational behaviour of cells expressing PctApp and various combinations of CheR and CheB activities was analysed. PctApp expression caused more than 70 % CW rotation in R^+B^+, R^-B^- and R^-B^+ strains, but only 40 % CW rotation in the R^+B^- strain. This result suggests that the unopposed methylation activity of CheR generates a receptor with reduced ability to activate CheA, in contrast to what is expected for a more methylated receptor. Even though more studies are needed to clarify this issue, our results thus far indicate that PctApp undergoes modification-dependent adaptation in *E. coli*.

Higher-order organization of PctApp in *E. coli*

E. coli MCPs of different specificities form trimers of dimers through multiple residue contacts in the highly conserved cytoplasmic tip (Kim *et al.*, 1999). The composition of those trimers reflects the relative abundance of the individual MCPs: abundant receptor types form pure trimers as well as mixed trimers with less abundant receptors (Studdert & Parkinson, 2004). Receptors carrying a cysteine reporter residue located immediately above the trimer contact region form trimer-derived two- and three-subunit cross-linking products in cells treated with TMEA, a trifunctional cross-linking reagent (Studdert & Parkinson, 2004). In the so-called competition assay (Fig. 6a), the high expression level of a second receptor lacking the cysteine reporter shifts many of the cysteine-bearing receptors into mixed trimers, causing a significant decrease in TMEA cross-linking products (Studdert & Parkinson, 2005). In contrast, a mutant receptor that cannot incorporate into trimers does not diminish TMEA cross-linking products. Thus, the competition assay distinguishes receptors that are able to incorporate into mixed trimers (competitors) from those that are not (non-competitors).

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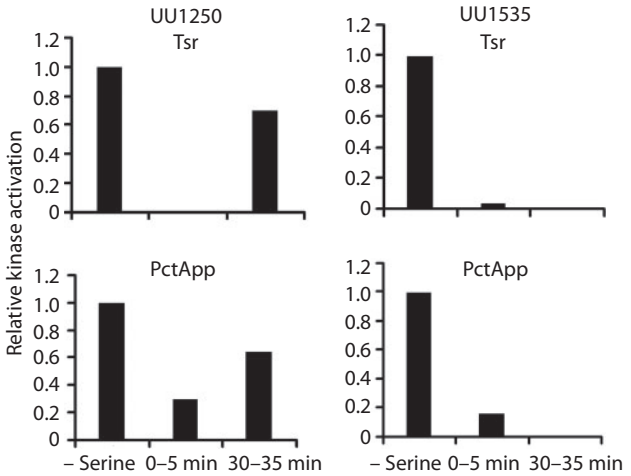

Fig. 5. Methylation-dependent adaptation. The rotational behaviour of cells expressing Tsr or PctApp was observed in the presence of tethering buffer and at different times after the addition of L-serine in the same buffer. Tsr-expressing cells were stimulated with 100 μM serine and PctApp-expressing cells with 700 μM serine. Relative kinase activation is represented by the percentage of CW rotation, calculated as described in Methods and normalized to the percentage CW observed for each strain in the presence of tethering buffer. The experiment was done in the absence of any other native receptor and in the presence (UU1250 cells, left) or absence (UU1535 cells, right) of the methylation enzymes CheR and CheB.

A competition assay was carried out to determine whether PctApp could incorporate into mixed trimers with Tar, the native *E. coli* aspartate receptor. Cells expressing a chromosomal copy of Tar-S364C were transformed with plasmids encoding PctApp or various forms of Tsr as controls, and their effect on Tar cross-linking products was evaluated. In the absence of any competitor, Tar cross-linking products formed efficiently upon TMEA treatment (Fig. 6b, vector control lanes). At high expression levels, Tsr-N376W, a trimer-proficient competitor, caused a clear decrease in Tar cross-linking products, as shown previously (Studdert & Parkinson, 2005) (Fig. 6b, N376W lanes). In contrast, neither Tsr-I377P, a trimer-deficient receptor (Studdert & Parkinson, 2005), nor PctApp caused any significant decrease in the abundance of Tar cross-linking products (Fig. 6b). These findings suggest that the foreign 40H-class receptor PctApp does not form mixed trimers efficiently with the native 36H-class Tar receptor.

To assess the localization of PctApp expressed in *E. coli*, fluorescence microscopy assays were performed using a CheZ–YFP reporter. The CheY-specific phosphatase CheZ localizes at the polar chemoreceptor clusters in *E. coli* through its interaction with the short version of CheA (CheAs; Cantwell *et al.*, 2003). We chose to use this reporter, as its polar localization reflects the assembly of CheA-containing ternary complexes.

When YFP–CheZ was expressed in UU9352 cells, lacking the chromosomal copies of the native MCPs and CheZ, the fluorescence was evenly distributed in the cells, indicating cytoplasmic localization of the CheZ–YFP fusion (Fig. 7, left panel). When Tsr was co-expressed in these cells, clear fluorescent spots were detected at the poles in around 70 % of the cells (Fig. 7, middle panel). The expression of PctApp also drove the polar localization of the YFP–CheZ fusion, albeit with reduced efficiency (Fig. 7, right panel). Only 30 % of the cells expressing PctApp and YFP–CheZ showed fluorescent spots at the poles, and the intensity of the spots was less than that for cells expressing Tsr. The co-expression of YFP–CheZ and Tsr or PctApp in cells lacking CheA and CheW did not generate any fluorescent spots at

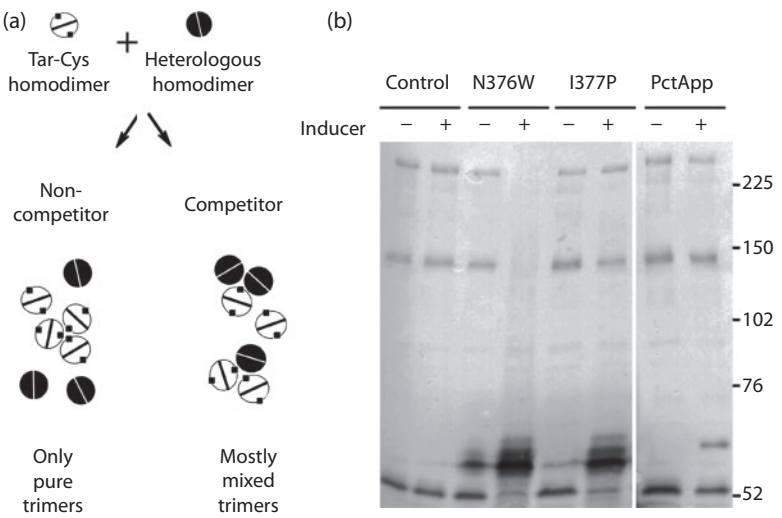

Fig. 6. Cross-linking assay to assess mixed-trimer formation. (a) Schematic representation of the competition assay. Circles divided by a middle line represent homodimers. Black dots on the circles indicate the presence of the reporter cysteine that is the TMEA target. (b) Competition assay with PctApp. UU1613 cells (expressing Tar-S364C as the only MCP and lacking CheA, CheW and methylation enzymes) were transformed with empty vector pKG116 (control), pCS12 N376W (N376W, trimer-proficient Tsr mutant), pCS12 I377P (I377P, trimer-deficient Tsr mutant) or pDSo1 (PctApp). MCPs were induced at 0 (–) or 1.2 μM (+) sodium salicylate, and cells were subjected to treatment with the tri-functional cross-linker TMEA as described in Methods. Samples were analysed by immunoblotting with anti-Tsr antibody.

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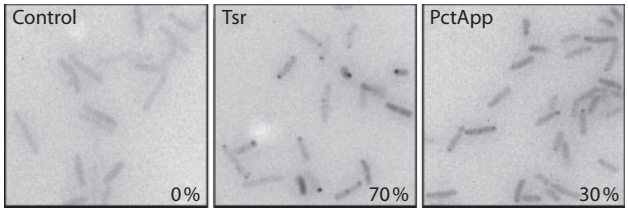

Fig. 7. PctApp localization. UU9352 cells (lacking all five native receptors and CheZ phosphatase) were co-transformed with pFG1 (YFP–CheZ) plus empty vector pKG116 (control), pCS12 (Tsr) or pDSo1 (PctApp). YFP–CheZ was induced at 100 μM IPTG and MCPs were induced at 0.6 μM sodium salicylate. Fluorescence pictures were obtained in a Nikon Eclipse Ti inverted microscope. Values at the bottom of the images indicate the percentage of cells with clear polar fluorescent spots.

the poles, confirming that polar localization of this CheZ reporter is dependent on the presence of properly assembled ternary complexes (data not shown).

YFP–CheZ localization in the presence of PctApp indicates that this receptor indeed forms ternary complexes that assemble into a properly localized chemoreceptor cluster when it is the only receptor in the cell.

DISCUSSION

PctApp signalling abilities within *E. coli* complexes

This study demonstrates that a foreign chemoreceptor, belonging to a length class different from *E. coli* MCPs, can mediate responses to specific stimuli. Even though the expression of PctApp, a putative amino acid receptor, did not confer chemotactic ability upon an *E. coli* strain lacking its native receptors (Fig. 3a), it did mediate motility responses to L-serine both in agarose-in-plug (Fig. 3b) and in tethered-cell assays (Fig. 4). The specificity of PctApp for different amino acids (Table 1) was similar to that established for PctA, its homologue from *P. aeruginosa*. The ability of PctApp to activate and control the *E. coli* CheA kinase implies that this foreign 40H-class MCP is able to assemble into functional ternary complexes with CheW and CheA, which normally partner with the 36H-class receptors of *E. coli*. Thus, there is no strict length-class specificity between receptors and the CheA kinase under their control. Likewise, the CheW coupling protein has been shown to be exchangeable between distantly related bacterial species (Alexandre & Zhulin, 2003; Hamblin *et al.*, 1997).

However, the inability to mediate proper chemotactic responses in semi-solid agar plates points to some defect in PctApp signalling in *E. coli*. The sensitivity of PctApp towards serine, as determined in tethered-cell assays, was about 30-fold lower than that of Tsr (Fig. 4b). Conceivably, such a reduced sensitivity accounts for the chemotaxis defect. In addition, we detected alterations in the adaptation response of PctApp (see below). A robust adaptation to attractant stimuli over a broad range of concentrations is presumably a *sine qua non* for the formation of the chemotactic rings in semi-solid agar plates. It is likely that PctApp-containing complexes do not fulfil this requirement.

Methylation-dependent adaptation to PctApp stimuli in *E. coli*

Cells expressing PctApp showed substantial sensory adaptation in flagellar rotation assays (Fig. 5, bottom-left panel). In the absence of the methylation enzymes, adaptation was completely abolished (Fig. 5, bottom-right panel). Both results were quite surprising, since we expected to find some incompatibility between the *E. coli* methylation enzymes and this 40H-class MCP. Sequence comparison between numerous 40H-class receptors failed to identify strongly conserved methylation sites, even though sites conforming to the established consensus {[ASTG]-[ASTG]-x-x-[EQ]-[EQ]-x-x-[ASTG]-[ASTG]} were found in a number of cases (Alexander & Zhulin, 2007). However, upon inspection of the PctApp amino acid sequence we did not find any site fully conforming to the consensus (Fig. 1b).

In vitro methylation assays using membranes prepared from *E. coli* cells that express PctA and purified CheR1 from *P. aeruginosa* have demonstrated that PctA is methylated by CheR1 methyltransferase at a rate that is increased by attractant stimuli (Schmidt *et al.*, 2011). However, those authors did not determine the sites at which the methylation occurred.

We examined the effect of the presence or absence of each of the methylation enzymes on the basal rotation behaviour of PctApp-expressing cells, and found that only CheR in the absence of CheB caused a significant rotational shift. Our interpretation is that *E. coli* CheR might methylate PctApp in non-canonical positions, and that this activity might be counterbalanced by CheB. The absence of CheB thus results in a more (aberrantly) methylated, less active receptor. In this scenario, it is difficult to explain the methylation-dependent adaptation that we observed in UU1250 cells. We speculate that the observed adaptation might depend on the deamidation activity of CheB, which can certainly be regulated by PctApp-modulated CheA. More experiments are needed to clarify this point.

PctApp higher-order organization and localization in *E. coli*

The competition cross-linking assay showed that overexpression of PctApp did not interfere with the formation of pure Tar TMEA cross-linking products (Fig. 6). This result indicates that PctApp cannot incorporate efficiently into mixed trimers with Tar. Taking into account the strong conservation of the protein interaction region (emphasized by the ability of PctApp to control *E. coli*

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CheA), we speculate that this inability to form mixed trimers is due to the difference in length between Tar (36H-class) and PctApp (40H-class) cytoplasmic domains.

We have previously demonstrated that Tar forms mixed trimers of dimers with two different 34H-class receptors from *Rhodobacter sphaeroides*, McpB and McpH, as well as with a Tsr-derived construct in which the deletion of a heptad in each arm of its cytoplasmic domain converts it into a 34H-like MCP (Massazza *et al.*, 2012). In those experiments, mixed trimer formation was accompanied by a significant impairment of Tar-mediated signalling, suggesting that a structural distortion in the trimers interferes with function. In Tar-only cells, expression of PctApp does not interfere with Tar function unless very high levels of PctApp are reached (data not shown), consistent with a failure to form mixed trimers of dimers.

It is becoming increasingly apparent that the organization of MCPs into trimer-of-dimer based clusters is a universal feature (Briegel *et al.*, 2009, 2012). Although PctApp was not able to form mixed trimers with receptors from a shorter class, it still remains to be examined in a direct way whether it does form trimers of dimers with receptors from the same length family.

Finally, we showed that PctApp was able to drive the polar localization of the CheZ–YFP reporter, although at a lower efficiency than did Tsr (Fig. 7). This observation suggests that PctApp promotes the assembly of complete chemoreceptor clusters.

E. coli as a system to study the specificity of foreign chemoreceptors

The present study shows that foreign chemoreceptors can function in *E. coli*. This could open the way to study the signalling behaviour of any MCP using *E. coli* cells as a model system. The vast MCP superfamily represents a wealth of chemical detection abilities, but only a tiny fraction of these chemoreceptors has been characterized. Knowledge of chemoeffectors specific to a given organism might provide crucial information for understanding the organism's biology and its interactions with other organisms. Our results highlight the possibility of using *E. coli* strains as reporters for the study of foreign receptors. Thus, the development of *E. coli*-based high-throughput methods for detection of CheA control would allow the study of stimuli responses by virtually any MCP.

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