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TlpC, a novel chemotaxis protein in *Rhodobacter sphaeroides*, localizes to a discrete region in the cytoplasm

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Summary

TlpC is encoded in the second chemotaxis operon of *Rhodobacter sphaeroides*. This protein shows some homology to membrane-spanning chemoreceptors of many bacterial species but, unlike these, is essential for *R. sphaeroides* chemotaxis to all compounds tested. Genomic replacement of *tlpC* with a C-terminal *gfp* fusion demonstrated that TlpC localized to a discrete cluster within the cytoplasm. Immunogold electron microscopy also showed that TlpC localized to a cytoplasmic electron-dense region. Correct TlpC–GFP localization depended on the downstream signalling proteins, $CheW_3$, $CheW_4$ and $CheA_2$, and was tightly linked to cell division. Newly divided cells contained a single cluster but, as the cell cycle progressed, a second cluster appeared close to the initial cluster. As elongation continued, these clusters moved apart so that, on septation, each daughter cell contained a single TlpC cluster. The data presented suggest that TlpC is either a cytoplasmic chemoreceptor responding to or integrating global signals of metabolic state or a novel and essential component of the chemotaxis signalling pathway. These data also suggest that clustering is essential for signalling and that a mechanism may exist for targeting and localizing proteins within the bacterial cytoplasm.

Introduction

The chemotaxis pathway allows bacteria to modulate their swimming behaviour so that they accumulate in environments that are beneficial for growth (for reviews, see Blair, 1995; Armitage, 1999). Motility and chemotaxis have been implicated in pathogenesis, symbiosis and biofilm development. For example, they are essential for the virulence and symbiosis of many medically important bacteria such as *Helicobacter pylori* and *Vibrio cholerae* and in the development of plant–bacterial relationships (Kim and Farrand, 1998; Klose and Mekalanos, 1998; Van de Broek *et al*., 1998; Kurdish *et al*., 2001; Watnick *et al*., 2001).

Chemotaxis has been studied extensively in *Escherichia coli.* This signal transduction pathway consists of four membrane-spanning methyl-accepting chemotaxis proteins (MCPs) that respond to a change in concentration of a limited number of periplasmic chemoeffectors to regulate the activity of a cytoplasmic histidine protein kinase CheA (for reviews, see Falke *et al*., 1997; Mowbray and Sandgren, 1998). A reduction in ligand occupancy of the appropriate MCP causes a conformational change across the membrane and results in autophosphorylation of the associated CheA via a linker protein CheW (Hess *et al*., 1988a,b). This phosphate moiety is transferred from CheA to two response regulators, CheY and CheB. Phosphorylated CheY binds to a component of the flagellar motor and results in switching (Welch *et al*., 1993). The signal from CheY-P is terminated by its spontaneous dephosphorylation, the rate of which is increased by CheZ.

MCPs from *E. coli* and a variety of other bacteria have a characteristic tertiary structure, with an N-terminal ligand-binding domain flanked by two transmembrane regions and a C-terminal highly conserved signalling domain. The signalling domain contains a region that interacts with CheW and CheA and two methylation regions that are involved in adaptation (Mowbray and Sandgren, 1998). Genome sequencing projects have shown that many non-enteric bacteria contain multiple genes that encode proteins homologous to MCPs, some of which are predicted to encode chemoreceptors that do not contain transmembrane regions (see http://www.tigr.org and http://www.jgi.doe.gov). One of these proteins (Car from *Halobacterium salinarum*) is responsible for taxis to arginine and is present in the cytoplasmic rather than the membrane fraction of the cell (Storch *et al*., 1999).

Adaptation is essential for chemotaxis, allowing bacte-

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Fig. 1. Alignment of 80 residues from the HCD of the classical chemoreceptors Tar and Tsr from *E. coli* and McpG from *R. sphaeroides*. TlpC and TlpS, two putative cytoplasmic chemoreceptors from *R. sphaeroides*, share some homology with the classical sequences but are less well conserved. The lower alignment, which compares only TlpC and TlpS, shows that these proteins share many of the differences in this region.

ria to respond to successive changes in attractant concentration. It involves the addition and removal of methyl groups from specific glutamate residues in the cytoplasmic domain of the MCPs, altering the level of activation of CheA. CheR is a constitutive methyltransferase that adds methyl groups from *S*-adenosyl methionine to these glutamate residues (Springer and Koshland, 1977). CheB is a methylesterase that removes these methyl groups, releasing them as methanol. The activity of CheB increases 10-fold on phosphorylation by CheA-P, a reaction that occurs at a slower rate than the phosphorylation of CheY (Hess *et al*., 1988b). The net result of these reactions is that the bacterium is able to respond to a change in attractant concentration by altering their swimming bias.

Immunogold electron microscopy and green fluorescent protein (GFP) techniques have shown that MCPs in all species so far examined form clusters at the poles of the cell (Alley *et al*., 1992; Maddock and Shapiro, 1993; Harrison *et al*., 1999; Gestwicki *et al*., 2000; Wadhams *et al*., 2000). In *E. coli*, this polar localization of MCPs is dependent on the presence of the cytoplasmic signal transduction proteins CheW and CheA. In the absence of either of these proteins, the MCPs are no longer polar but appear diffuse throughout the membrane (Maddock and Shapiro, 1993).

Rhodobacter sphaeroides, an α-subgroup purple, non-sulphur photosynthetic bacterium, has multiple copies of most of the *E. coli* chemotaxis genes, predominantly encoded in three chemotaxis operons. Transport and partial metabolism are required for some chemosensory responses (Jeziore-Sassoon *et al*., 1998), and the responses generated can depend on the growth conditions (Shah *et al*., 2000). *R. sphaeroides* also contains 13 genes that show homology to *MCPs*. Of these, McpG has been shown to localize to a pole of the cell (Wadhams *et al*., 2000). This localization is dependent upon the presence of $CheW_2$ and $CheA_2$. $CheW_3$ is also essential under photoheterotrophic, but not aerobic, conditions (Martin *et al*., 2001a). These three proteins are all encoded in *che*Op_2. The reason for the localization of MCPs to a polar cluster is unknown. It has been suggested that clustering may be essential for signal generation, amplification and/or adaptation (Bray *et al*., 1998; Levit *et al*., 1998).

At the 3′ end of *che*Op_2 is a gene, *tlpC*, that encodes a protein that shows limited homology to the highly conserved domain of MCPs, but has no obvious transmembrane regions or methylation sites (Hamblin *et al*., 1997a). A similar gene, *tlpS*, has been found upstream of *che*Op_1. The aim of this study was to characterize the role of TlpC in *R. sphaeroides* chemotaxis and to examine the localization of this novel chemotaxis protein within the *R. sphaeroides* cell.

Results

Alignment with other chemoreceptors

tlpC is predicted to encode a 61.6 kDa protein with a pI of 5.6. A BLAST search using the predicted TlpC sequence identified MCPs as the only proteins in the SWISSPROT and TrEMBL databases sharing significant identity. The alignment shows ≈80 residues from the highly conserved domain (HCD) of *E. coli* Tar and Tsr and McpG from *R. sphaeroides* that are extremely well conserved in all characterized MCPs (Fig. 1). However, the predicted TlpC and TlpS sequences, although showing homology to this region, are considerably less well conserved. Interestingly, many of the differences seen in this region are conserved between TlpC and TlpS. Sequence analysis using HMMTOP (http://www.enzim.hu/hmmtop/index.html) did not identify any transmembrane domains, and there are no putative methylation site consensus sequences that are characteristic of classical MCPs.

Phenotypic analysis of a ΔtlpC mutant

To investigate the role of TlpC in chemotaxis, an unmarked, internal, in frame deletion of *tlpC* was generated (JPA470). The ability of this strain to respond to chemoeffectors was assessed both on swarm plates and in the tethered cell assay. Swarm plates are sloppy agar plates containing chemoattractant, which the bacteria metabolizes creating a concentration gradient out from the

inoculation site. Chemotactic bacteria swim up this concentration gradient, forming a swarm ring. The swarm diameters generated by the mutant to all compounds tested under both aerobic and photoheterotrophic conditions were significantly reduced with respect to the wild type ($P < 0.05$) (Fig. 2). These differences were not the result of changes in the growth rate of the mutant, which was normal on the carbon sources tested (data not shown). The small swarms formed by the wild type on a 'no added attractant' control plate were presumed to result from responses to trace nutrients in the agar. The slightly increased size of mutant swarms was the result of growth on the carbon source, not chemotaxis.

To examine the responses of individual cells, they were tethered by their flagella in a flow cell, and changes in the rotation rate of their flagella were assessed by observing the counter-rotation of the cell body on the addition or removal of attractant. The wild-type response was characterized by a slight increase in the apparent rotation rate of the cells in response to the addition of 1 mM propionate. Upon removal of the attractant, the cells showed a prolonged stop followed by adaptation back to prestimulus behaviour (Fig. 3A and B). Deletion of *tlpC* resulted in a strain that showed no response to either the addition or the removal of propionate (Fig. 3C and D) but still retained a wild-type stopping frequency (data not shown).

Complementation of the deletion strain by a plasmid containing *tlpC*, pCΩ1, restored chemotaxis to propionate under aerobic conditions as assessed both on swarm plates and in the tethered cell assay (data not shown). This demonstrates that the phenotype of the Δ*tlpC* mutant was the direct result of the deletion of the *tlpC* gene. Taken together, these data show that TlpC is essential for chemotaxis to all the compounds tested.

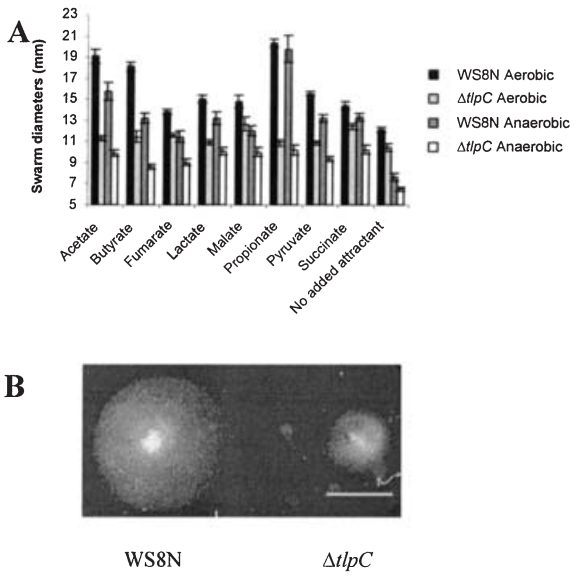

Fig. 2. A. Comparison of the swarm diameters of the wild-type (WS8N) and the deletion mutant (JPA470) under aerobic and photoheterotrophic conditions to 100 μM attractants. The error bars represent the standard error of the mean from nine experiments. The Δ*tlpC* mutant has significantly reduced swarm diameters to all attractants tested under both environmental conditions ($P < 0.05$).
B. Swarm plate of WS8N and the Δ*tlpC* mutant showing responses to 100 μM propionate under aerobic conditions. The bar represents 1 cm.

TlpC is not involved in methanol release

In *E. coli*, CheB-P releases methyl groups from the MCPs as methanol during adaptation. Hence, methanol is released upon the removal of attractant because of the increased activity of CheB upon phosphorylation by CheA-P. However, in *Bacillus subtilis*, methanol release is observed on both the addition and the removal of attractants (Kirby *et al*., 1997). We have shown previously that *R. sphaeroides* releases measurable methanol only upon the addition of attractant (Martin *et al*., 2001b) (Fig. 4A). To investigate whether signalling via TlpC required receptor adaptation and methylation, the methanol release profile of the Δ*tlpC* strain to the addition and removal of 1 mM propionate was investigated. As in the wild type, methanol was released upon propionate addition, but not upon its removal (Fig. 4B). This demonstrates that methylation-dependent adaptation to propionate is unaffected in the Δ*tlpC* strain, even though there was no chemotactic response to propionate.

TlpC is a cytoplasmic chemotaxis protein

To examine the location of TlpC within the cell, a C-terminal GFP fusion construct was introduced into the wild-type genome, replacing *tlpC* with *tlpC–gfp* (JPA543). The fusion protein was expressed from a single copy in the genome and was thus under the same regulation as the wild-type gene. Although the presence of the GFP tag reduced the ability of *R. sphaeroides* to respond to shallow gradients of chemoeffectors on swarm plates, the strain showed normal responses to 1 mM propionate in tethered cell assays (data not shown). Thus, TlpC–GFP is partially functional in chemotaxis. No fluorescence was observed from the wild-type strain (WS8N) in the absence of the GFP tag (data not shown). TlpC–GFP localized predominantly to a single cluster within the cytoplasm of the *R. sphaeroides* cell (Fig. 5A). Fluorescence associated with the cell membrane was never observed. In general, it has been assumed that bacterial cells do not contain subcellular organelles or show subcellular organization. However, these results show that TlpC is targeted to a specific region of the cytoplasm.

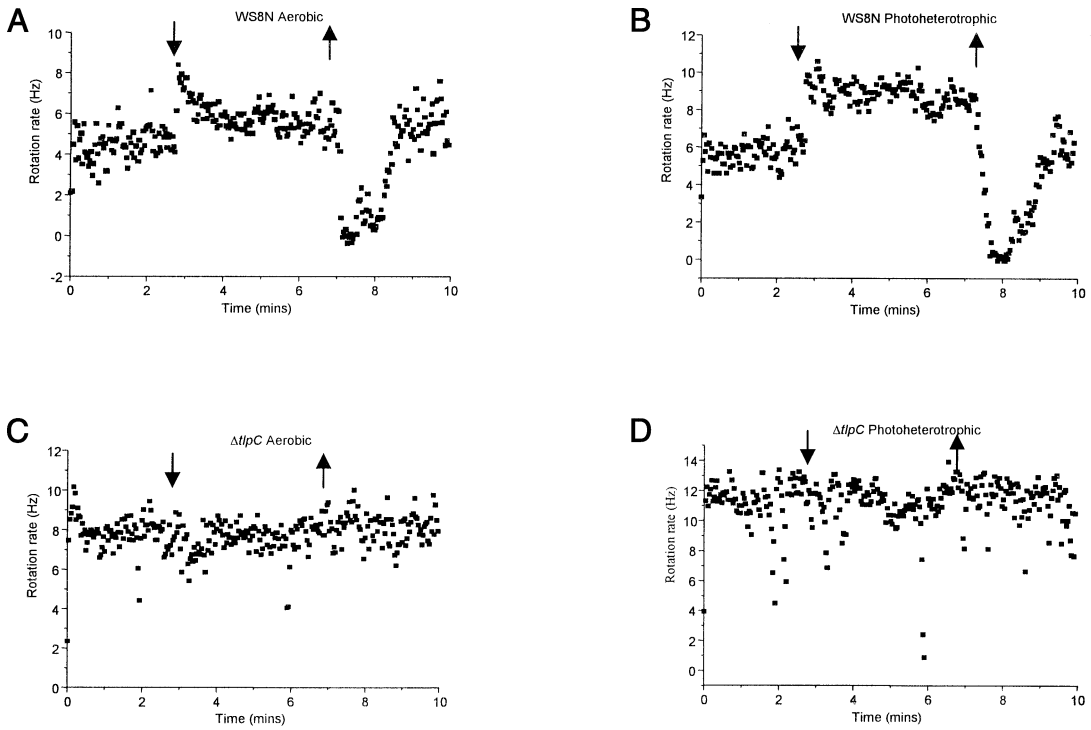

Fig. 3. The behaviour of the wild type (WS8N) and the Δ*tlpC* mutant in the tethered cell assay. The strains and the growth conditions are indicated above each graph. The addition and removal of 1 mM propionate is shown by arrows. The results are the average of at least 10 cells. The apparent increase and decrease in rotation rate represents increased smooth swimming and stopping respectively.

TlpC is part of an electron-dense region

To confirm that the localization of the partially functional TlpC–GFP-tagged protein is representative of the wild-type protein, and to counter the possibility that the GFP fluorescence may have originated from an inclusion body, we also used electron microscopy to demonstrate that TlpC is targeted to a specific region of the cytoplasm.

A rabbit polyclonal antibody was generated to His-tagged TlpC and immunodepleted with acetone powders prepared from the Δ*tlpC* strain JPA470. Western blot analysis demonstrated that this serum contained antibody that reacted strongly with pure TlpC protein and with a protein of the same molecular weight as TlpC that was present in WS8N but was absent from the Δ*tlpC* mutant (data not shown).

The localization of gold particles was assessed in 160 randomly selected dividing cells by immunogold electron microscopy with this immunodepleted antibody. It was demonstrated conclusively that TlpC is a cytoplasmic protein and is not membrane associated (Fig. 5B, Table 1). There were an average of six gold particles per cell, and 88% of the signal was from the cytoplasm. Of the cytoplasmic signal, a significant proportion (73% of the total) originated from an uncharacterized electron-dense region (EDR). By comparison, there was very little background signal in the Δ*tlpC* strain with only 1.1 gold particles per cell. The composition of the EDR is unknown, but it was observed in an earlier study using anti-Tsr antibody (Harrison *et al.*, 1999).

TlpC–GFP localization is linked to the cell cycle

The pattern of TlpC–GFP fluorescence through the cell cycle was assessed by time-lapse imaging of actively dividing cells. Just before cell division, a second point of fluorescence was observed adjacent to the original TlpC–GFP cluster (Fig. 6). As division continued, these two clusters moved apart so that, on division, each daughter cell contained a single fluorescent locus. This demonstrates that the number and position of the TlpC clusters are tightly controlled in *R. sphaeroides* and are linked to the cell cycle.

Requirement for CheWs and CheAs in TlpC localization

In *E. coli*, the normal polar localization of MCPs is dependent on the presence of the downstream signalling proteins CheW and CheA (Maddock and Shapiro, 1993). Previously, we have demonstrated that the correct polar localization of McpG from *R. sphaeroides* is dependent on $CheW_2$, $CheW_3$ and $CheA_2$ (Wadhams *et al.*, 2000). *R.*

Table 1. Spatial distribution of TlpC by immunogold electron microscopy.

Strain	Total cells	Total particles	Mean particles per cell	Cyto. particles	EDR-associated particles	Clustered particles in EDR	% cyto.	% EDR associated	% clustered in EDR
WS8N	160	963	6.0	851	699	507	88.4	72.6	52.6
JPA470	160	175	1.1	97	29	0	55.4	16.6	0

All percentages are with respect to the total number of particles. The majority of the gold particles were in the cytoplasm (cyto.) of the wild-type strain (WS8N). Of the cytoplasmic signal, the majority of particles were associated with an electron-dense region (EDR). The background signal from the Δ*tlpC* control strain (JPA470) was very low.

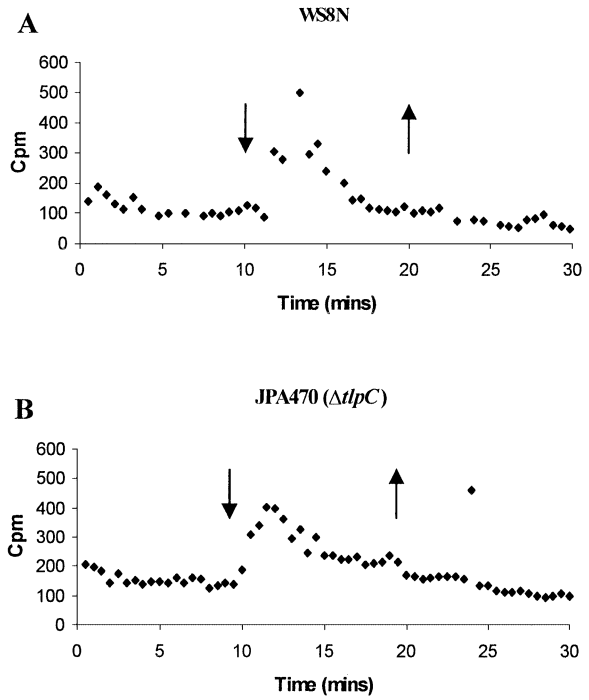

Fig. 4. Methanol release from (A) wild-type (WS8N) and (B) Δ*tlpC* mutant (JPA470) strains upon the addition and removal of 1 mM propionate (shown by arrows). In both strains, methanol was released upon propionate addition, but not upon its removal. This demonstrates that methylation-dependent adaptation to propionate is unaffected in the Δ*tlpC* strain.

sphaeroides has four putative CheA and four putative CheW homologues encoded in three *che* operons (Porter *et al.*, 2002). To investigate the requirement, if any, for these proteins in the correct localization of TlpC, strains were generated in which the fusion construct pK18tlpCgfp was introduced into each of the *cheW* and *cheA* deletion strains (Table 2).

The fluorescence patterns of these mutant strains were compared with that of the wild-type fusion strain. The presence or absence of a discrete point of fluorescence was recorded, and its intensity was scored on a scale ranging from + (low fluorescence intensity) to +++++ (high fluorescence intensity). In the absence of $CheW_1$, $CheW_2$, $CheA_1$, $CheA_3$ or $CheA_4$, there was no difference in the localization of TlpC–GFP (Table 2). However, deletion of either $CheW_3$ or $CheW_4$ resulted in a dramatic reduction in the localized signal, with more fluorescence visible throughout the whole cytoplasm (Fig. 7, Table 2). Interestingly, the absence of $CheA_2$ resulted in an intermediate phenotype in which the cytoplasmic cluster was still visible, although the level of fluorescence was considerably weaker. Quantitative immunoblots with the antibody to TlpC showed that the level of TlpC protein in the cell was unchanged in the Δ*cheW*$_3$, Δ*cheW*$_4$ and Δ*cheA*$_2$ mutants (data not shown).

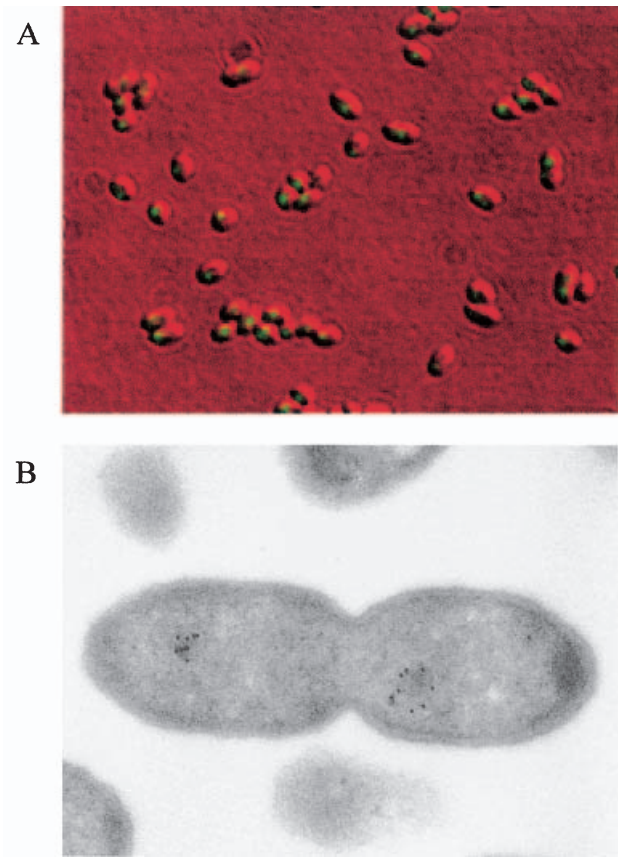

Fig. 5. A. Localization of TlpC–GFP to a discrete point in the cytoplasm in the genomic replacement, fusion strain JPA543.
B. Immunogold electron microscopy using an antibody to TlpC showing TlpC localized to an electron-dense region in the cytoplasm of wild-type cells.

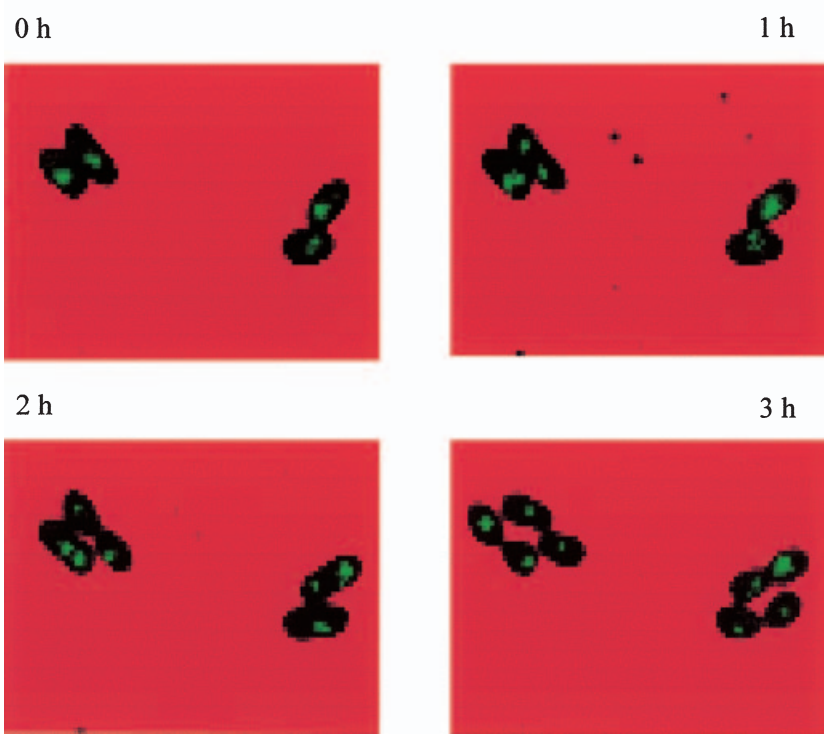

Fig. 6. Time course of TlpC–GFP localization in cells embedded in 0.8% succinate agarose. Images were collected at hourly intervals. Before cell division, a second point of fluorescence was observed adjacent to the original TlpC–GFP cluster. These two clusters moved apart as division continued so that, on septation, each daughter cell contained a single fluorescent locus.

Discussion

Here, we have demonstrated by both GFP fusions and immunogold electron microscopy that TlpC, a protein essential for normal *R. sphaeroides* chemotaxis, is localized to a discrete region of the cytoplasm close to the centre of the cell. The constituents and function of this region, visualized as an electron-dense region (EDR) in electron micrographs, are currently unknown. However, an antibody to the highly conserved domain of *E. coli* Tsr was also shown by immunogold electron microscopy to cross-react with proteins in this region (Harrison *et al*., 1999). Cross-reaction with the anti-Tsr antibody was still measurable in the Δ*tlpC* strain, implying that the EDR may also contain other *R. sphaeroides* Tlps and that the presence of TlpC is not required for EDR formation (unpublished observations). A third putative cytoplasmic chemotaxis protein, TlpT, was identified in the third recently identified chemotaxis locus in *R. sphaeroides* (Porter *et al*., 2002). This is also needed for normal chemosensory responses and may therefore form part of the cluster. TlpS appears to be expressed at very low levels, if at all, under normal laboratory conditions, and its deletion has no effect on behaviour, and no fluorescence was seen in a strain carrying a *tlpS–gfp* fusion (unpublished data). The sequencing of the complete genome has identified a gene encoding a fourth possible Tlp-like protein, TlpL. It therefore seems possible that this EDR comprises a cytoplasmic cluster of proteins that probably includes multiple cytoplasmic chemoreceptors as well as TlpC, analogous to the mixed clusters of receptors seen at the poles of *E. coli*.

Clustering of transmembrane chemoreceptors has been implicated in signal generation, amplification and adaptation (Bray *et al*., 1998; Levit *et al*., 1998). It seems possible therefore that the clustering of TlpC may be a prerequisite for its function, as has been assumed for classical MCPs. To the best of the authors' knowledge, these data represent the first direct evidence for the cytoplasmic subcellular localization of a bacterial protein with no identifiable function in DNA replication or cell division. This protein also demonstrates a degree of organization

Table 2. TlpC–GFP localization in strains deleted for each of the *cheW* and *cheA* homologues.

Strain	TlpC localization	Relative fluorescence intensity
JPA543 (wild type)	Yes	+++++
JPA557 (Δ$cheA_1$)	Yes	+++++
JPA548 (Δ$cheA_2$)	Yes	+++
JPA1317 (Δ$cheA_3$)	Yes	+++++
JPA1311 (Δ$cheA_4$)	Yes	+++++
JPA549 (Δ$cheW_1$)	Yes	+++++
JPA573 (Δ$cheW_2$)	Yes	+++++
JPA550 (Δ$cheW_3$)	No	+
JPA1327 (Δ$cheW_4$)	No	+

The presence or absence of a discrete point of fluorescence was recorded and its intensity scored on a scale ranging from + (low fluorescence intensity) to +++++ (high fluorescence intensity).

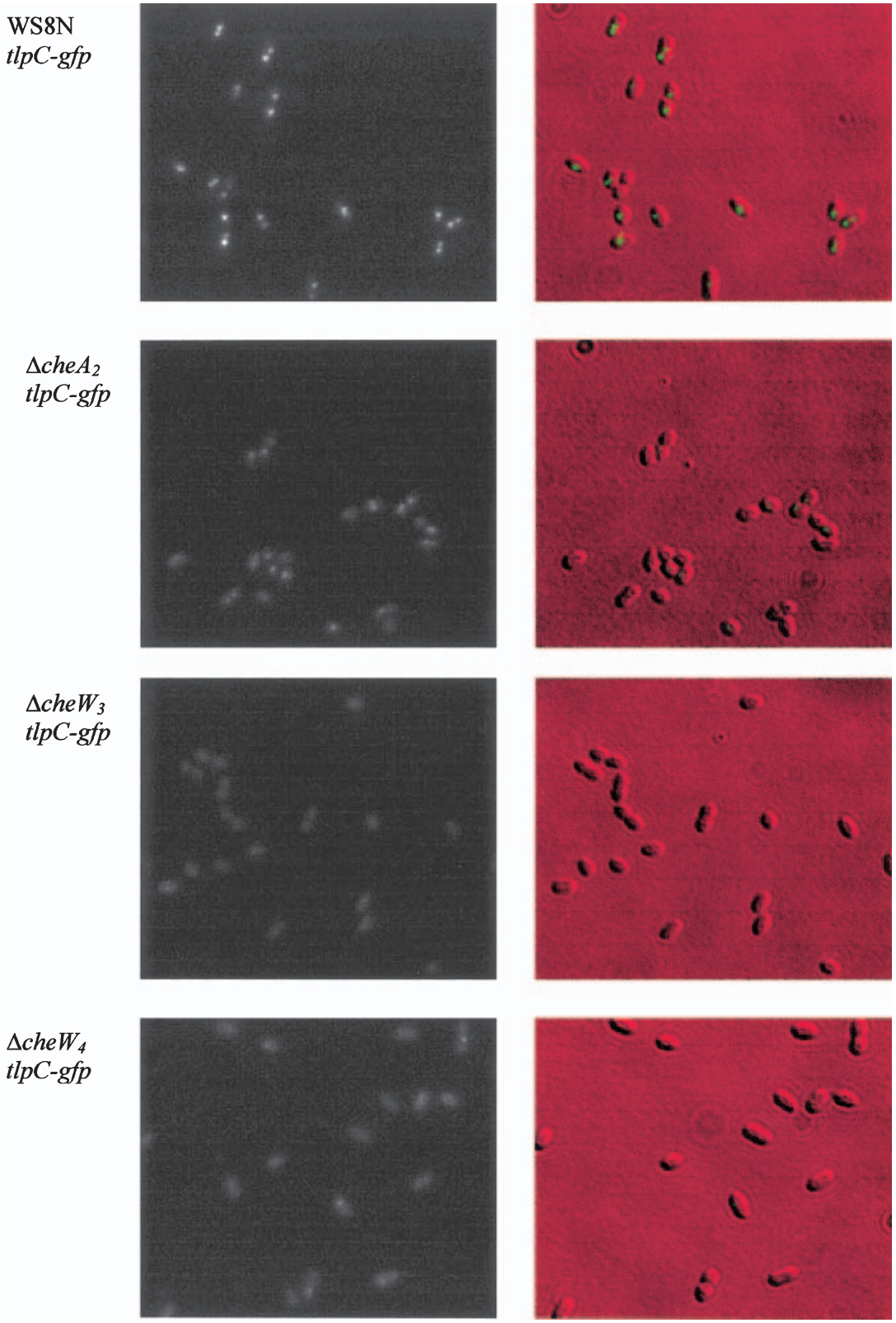

Fig. 7. TlpC–GFP localization in cells deleted for *cheA*$_2$ (JPA548), *cheW*$_3$ (JPA550) or *cheW*$_4$ (JPA1327). Deletion of either CheW$_3$ or CheW$_4$ resulted in a dramatic reduction in the localized signal, with more fluorescence visible throughout the whole cytoplasm. The absence of CheA$_2$ resulted in an intermediate phenotype in which the cytoplasmic cluster was visible, but the fluorescence was less intense.

within the bacterial cytoplasm not seen previously for an environmental sensing or signalling protein.

The polar clustering of MCPs in many bacterial species has been shown to be dependent on the presence of certain CheW and CheA homologues (Maddock and Shapiro, 1993; Martin *et al*., 2001a). With this in mind, we examined the requirement for each of the *R. sphaeroides* CheW and CheA homologues for the correct localization of TlpC. Quantitative immunoblots were used to demonstrate that the deletion of *cheW* and *cheA* homologues did not affect the level of expression of TlpC. GFP localization experiments demonstrated a requirement for $CheW_3$, $CheW_4$ and, to some extent, $CheA_2$ for the correct localization of TlpC to a cytoplasmic cluster. Whether this requirement results from the direct interaction of these CheW and CheA proteins with TlpC or whether they interact with other components of the EDR remains to be determined. However, in the absence of $CheA_2$, localization was only slightly reduced, whereas in the absence of either $CheW_3$ or $CheW_4$, TlpC localization was significantly impaired, suggesting a major role for two of the four *R. sphaeroides* CheW proteins in creating or holding the cluster together. Interestingly, these CheW proteins are encoded on different *che* operons, both of which also encode putative Tlp proteins. Deletion of either $CheA_2$ or $CheW_4$ results in the loss of chemotaxis. Whether this is the result of the loss of signalling or the loss of integrity of the clusters is unknown. Preliminary data indicate that, although $CheA_2$ is predominantly associated with the polar cluster of chemoreceptors, $CheW_4$ localizes to the cytoplasmic cluster.

The localization of TlpC was tightly controlled within the cell, with almost all cells having a single cluster at about the mid-point. Analysis of actively dividing cells demonstrated that the location and number of these clusters was tightly linked to the cell cycle. Just before cell division, a second cluster appeared, and the two resulting clusters moved apart so that, on septation, both daughter cells contained a single TlpC cluster. It is unclear what controls the formation of a second cluster and how partitioning is regulated with respect to the cell cycle. Although it is not possible to rule out the possibility that TlpC is either directly or indirectly associated with the DNA and that its localization is governed by DNA replication, we have been unable to identify any DNA-binding motifs within the protein sequence or to isolate DNA in association with the TlpC cluster (unpublished observations). Whatever mechanism is responsible for this precise control over TlpC localization, these data suggest that the cluster size and its location may be essential for optimum chemotaxis.

In contrast to all putative MCPs analysed so far in *R. sphaeroides*, deletion of *tlpC* resulted in the abolition of chemotaxis to all compounds tested under both aerobic and photoheterotrophic growth conditions. Despite the fact that deletion of *tlpC* resulted in a strain that was unable to respond to propionate either on swarm plates or in the tethered cell assay, the methanol release profile of this strain in response to propionate was normal. This implies that, although propionate is being sensed and the adaptation pathway is functional, deletion of *tlpC* prevents this signal from being transduced to the motor. Therefore, the activity of the CheW, CheA, CheR and CheB homologues that are required for the adaptation response to propionate must be unaffected by the *tlpC* deletion.

TlpC, along with TlpS from *R. sphaeroides*, shows some homology to the highly conserved domain of classical MCPs. However, they have no putative transmembrane regions and do not have any consensus methylation sites. This is unlike the cytoplasmic chemoreceptor Car from *H. salinarum* (Storch *et al*., 1999), and two other Tlps identified in *R. sphaeroides*, TlpT and TlpL (http://www.jgi.doe.gov/JGI_microbial/html/rhodobacter/rhodob_homepage.html), which have strong homology with the highly conserved domain of *E. coli* MCPs and appear to have methylation sites.

Here, we have characterized the novel chemotaxis protein TlpC from *R. sphaeroides*. The data presented here lead us to suggest that TlpC is either a cytoplasmic chemoreceptor responding to or integrating a global signal of metabolic state or a novel and essential component of the *R. sphaeroides* chemotaxis signalling pathway. Whatever the precise role of TlpC, this essential component of the *R. sphaeroides* chemotaxis signalling pathway is providing a new insight into the cytoplasmic organization and protein partitioning of bacterial cells. In addition to proteins that participate in cell division and DNA segregation, this study suggests that bacterial cells also tightly regulate the localization and segregation of essential signalling proteins.

Experimental procedures

Strains and growth conditions

Bacterial strains and plasmids are listed in Tables 3 and 4 respectively. *R. sphaeroides* strains were grown aerobically in succinate medium (Sistrom, 1960) at 30°C with shaking. *E. coli* strains DH5α and S17-1 λ*pir* were grown in Luria–Bertani (LB) medium at 37°C. When appropriate, the antibiotics nalidixic acid, kanamycin and streptomycin were used at 25 μg ml^{-1} and ampicillin at 100 μg ml^{-1}.

Molecular genetic techniques

All cloning steps were performed by standard methods (Sambrook and Russell, 2001). Polymerase chain reactions (PCRs) were performed with *Pfu* DNA polymerase (Promega) as advised by the manufacturer. Sequencing quality DNA was prepared using the Plasmid midi kit (Qiagen), sequenced by the University of Oxford Biochemis-

try sequencing service and analysed with the GCG software package (University of Wisconsin). All primers were supplied by Genosys Biotechnologies.

Construction of deletion

A 1.3 kb *Sal*I–*Stu*I fragment from pJPA114 and a 0.6 kb *Stu*I–*Bgl*II fragment from pJPA135 were cloned together into a *Sal*I–*Bam*HI-cut pUC19 to generate pHMK1.8. This construct contained the N- and C-termini of *tlpC* deleted between bases 272 and 1496 with the join in frame, together with upstream and downstream flanking regions. The whole insert from this plasmid was excised and cloned into pK18*mobsacB*. The construct was sequenced to ensure that the regions were in frame and contained no errors. The mutation was introduced into the genome by allelic exchange as described previously (Schäfer *et al*., 1994; Hamblin *et al*., 1997b). The correct insertion was confirmed by Southern blot, PCR and sequencing.

Behavioural assays

Swarm plates containing 0.25% agar (BiTek, Difco) and 100 μM attractant in M22 media were inoculated, incubated and analysed as described elsewhere (Martin *et al*., 2001a). Each experiment was performed in triplicate and repeated three times to generate nine data sets.

Aerobically grown cells were tethered in a flow chamber, and responses to the addition and removal of 1 mM propionate were examined as described previously (Martin *et al*., 2001a). At least three data sets, which together included at least 10 cells, were collected. Growth rates were measured as described by Martin *et al*. (2001a).

For complementation studies, *tlpC* was amplified by PCR with primers that included a 5′ *Eco*RI site and a 3′ *Hin*dIII site to facilitate cloning into pRK415. A Sm^R Ω cartridge from pHP45Ω was inserted into the *Eco*RI site of both pRK415 and the *tlpC*-containing derivative to generate pRKΩ1 and pCΩ1 respectively. These were conjugated into WS8N and JPA470, and chemotaxis assays were performed with the resultant strains as described above.

Methanol release experiments

Methanol release experiments were performed as described in detail previously (Martin *et al*., 2001b). At least three data sets were collected for each strain.

Construction of gfp *fusion strains*

A region immediately downstream from *tlpC* was amplified by PCR to include a 5′ *Xba*I site and a 3′ *Eco*RI site and cloned into pUC19 to generate ptlpCd. *egfp* was excised from pEGFP-N1 (Clontech) with *Pst*I and *Xba*I and ligated into ptlpCd to form ptlpCmd. *tlpC* was amplified by PCR to include a 5′ *Hin*dIII site and a 3′ *Nsi*I site. This fragment was cloned into ptlpCmd, which had been linearized with *Hin*dIII and *Pst*I (*Nsi*I and *Pst*I generate complementary cohesive ends) to generate a plasmid containing an in frame *tlpC–gfp* fusion and downstream flanking region, ptlpCgfp. The whole construct was then excised from pUC19 and ligated into the suicide vector pK18*mobsac*B with *Eco*RI and *Hin*dIII to generate pK18tlpCgfp. This construct was inserted into WS8N and the appropriate *cheW* and *cheA* deletion strains (Table 3) by allelic exchange as described previously (Schäfer *et al*., 1994; Hamblin *et al*., 1997b). Deletion of $cheA_3$, $cheW_4$ and $cheA_4$ was performed as follows. A region immediately down-

Table 3. Strains used in this study.

Strain	Characteristics	Source
R. sphaeroides WS8N	A spontaneous nalidixic acid-resistant mutant of wild-type WS8	Sockett *et al*. (1990)
R. sphaeroides JPA470	Δ*tlpC* derivative of WS8N	This study
R. sphaeroides JPA543	WS8N containing a *tlpC–gfp* fusion in place of the wild-type *tlpC* in the chromosome	This study
R. sphaeroides JPA120	Δ$cheW_1$ derivative of WS8N	Hamblin *et al*. (1997b)
R. sphaeroides JPA514	Δ$cheW_2$ derivative of WS8N	Martin *et al*. (2001a)
R. sphaeroides JPA527	Δ$cheW_3$ derivative of WS8N	Martin *et al*. (2001a)
R. sphaeroides JPA1325	Δ$cheW_4$ derivative of WS8N	This study
R. sphaeroides JPA525	Δ$cheA_1$ derivative of WS8N	Martin *et al*. (2001a)
R. sphaeroides JPA211	Δ$cheA_2$ derivative of WS8N	Hamblin *et al*. (1997a)
R. sphaeroides JPA1314	Δ$cheA_3$ derivative of WS8N	This study
R. sphaeroides JPA1308	Δ$cheA_4$ derivative of WS8N	This study
R. sphaeroides JPA557	Δ$cheA_1$ derivative of JPA543	This study
R. sphaeroides JPA548	Δ$cheA_2$ derivative of JPA543	This study
R. sphaeroides JPA1317	Δ$cheA_3$ derivative of JPA543	This study
R. sphaeroides JPA1311	Δ$cheA_4$ derivative of JPA543	This study
R. sphaeroides JPA549	Δ$cheW_1$ derivative of JPA543	This study
R. sphaeroides JPA573	Δ$cheW_2$ derivative of JPA543	This study
R. sphaeroides JPA550	Δ$cheW_3$ derivative of JPA543	This study
R. sphaeroides JPA1327	Δ$cheW_4$ derivative of JPA543	This study
E. coli DH5α	Cloning strain that supports blue/white screening of colonies	Gibco BRL
E. coli S17-1 λ*pir*	A strain capable of mobilizing the suicide vector pK18*mobsacB* into *R. sphaeroides*, Sm^R	Penfold and Pemberton (1992)

Table 4. Plasmids used in this study.

Plasmid	Characteristics	Source
pUC19/18	High-copy-number cloning vector, Ap^R	Pharmacia
pJPA114	A 4.8 kb *Bam*HI fragment from *R. sphaeroides che*Op_2, in pUC19	Hamblin *et al.* (1997a)
pJPA135	A 1.3 kb *Bam*HI fragment from *R. sphaeroides che*Op_2, in pUC18	Hamblin *et al.* (1997a)
pHMK1.8	A *Sal*I–*Stu*I fragment from pJPA114 joined in frame with a *Stu*I–*Bgl*II fragment from pJPA135 in pUC19	This study
pRK415	Low-copy-number, broad-host-range vector, Tc^R	Keen *et al.* (1988)
pHP45Ω	Plasmid that carries the omega fragment, Ap^R, Sm^R	Prentki and Krisch (1984)
pRKΩ1	Derivative of pRK415 containing the Ω fragment that confers Sm^R	This study
pCΩ1	Derivative of pRKtlpC containing the Ω fragment that confers Sm^R	This study
pEGFP-N1	GFP fusion vector, Km^R	Clontech
ptlpCd	An *Xba*I–*Eco*RI PCR fragment containing the downstream flanking region of *tlpC* cloned into pUC19	This study
ptlpCmd	*egfp* excised from pEGFP-N1 as a *Pst*I–*Xba*I fragment and ligated into ptlpCd to form ptlpCmd	This study
ptlpCgfp	A *Hin*dIII–*Nsi*I PCR fragment containing *tlpC* cloned into ptlpCmd to generate an in frame *tlpC–gfp* fusion with downstream flanking region	This study
pK18*mobsacB*	Allelic exchange suicide vector mobilised by *E. coli* S17-1 λ*pir*	Schäfer *et al.* (1994)
pK18tlpCgfp	*tlpC–gfp* fusion construct and downstream flanking sequence from ptlpCgfp in pK18*mobsacB*	This study

stream of each gene was amplified by PCR to include a 5′ *Xba*I site and a 3′ *Hin*dIII site. A region immediately upstream of each gene was amplified by PCR to include either a 5′ *Mfe*I site (*cheA3*) or a 5′ *Eco*RI site (*cheW4* and *cheA4*) and a 3′ *Xba*I site. These were ligated in frame into pK18*mobsacB* to generate deletion constructs that were inserted into WS8N by allelic exchange.

Fluorescence analysis

Log-phase cultures were embedded in 1.2% agarose on microscope slides. Differential interference contrast (DIC) and fluorescence images were acquired using a Nikon TE200 microscope with a GFP filter set and recorded with a cooled CCD camera (Hamamatsu). The images were superimposed with IMAGE ANALYSIS software from Digital Pixel. For analysis of the deletion mutants, the presence or absence of a discrete point of fluorescence was recorded, and its intensity was scored by eye on a scale ranging from + (low fluorescence intensity) to +++++ (high fluorescence intensity). The whole field of view was considered, and results were collected from five independent cultures for each strain.

For analysis of localization through the cell cycle, cultures were embedded in 0.8% succinate agarose, and brightfield and fluorescence images taken as described previously (Wadhams *et al.*, 2000).

Immunogold electron microscopy

Motile aerobic cultures of *R. sphaeroides* were fixed, embedded in resin, sectioned and placed on nickel grids as described previously (Harrison *et al.*, 1999). An antibody that had been raised against purified TlpC (Eurogentec) was adsorbed with acetone powders prepared from JPA470 by standard methods (Harlow and Lane, 1988). Antibody reactions and electron microscopy were performed as described previously (Harrison *et al.*, 1999) using primary antibody diluted 1:200 and secondary antibody (12 nm colloidal gold particles conjugated to goat antibody to rabbit IgG; Jackson Immunoresearch) diluted 1:40.

Particles were scored as being either cytoplasmic or membrane associated. It was noted whether those particles that were cytoplasmic were also associated with the EDR and whether they clustered. A cluster was defined as three or more gold particles each located no more than 20 nm from its neighbour together with any outlying particles that were no more than 40 nm from the core cluster.

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References

Alley, M.R.K., Maddock, R., and Shapiro, L. (1992) Polar localization of a bacterial chemoreceptor. *Genes Dev* **6:** 825–836.

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

Blair, D.F. (1995) How bacteria sense and swim. *Annu Rev Microbiol* **49:** 489–522.

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

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

Gestwicki, J.E., Lamanna, A.C., Harshey, R.M., McCarter, L.L., Kiessling, L.L., and Adler, J. (2000) Evolutionary conservation of methyl-accepting chemotaxis protein location in Bacteria and Archaea. *J Bacteriol* **182:** 6499–6502.

Hamblin, P.A., Maguire, B.A., Grishanin, R.N., and Armitage, J.P. (1997a) Evidence for two chemosensory pathways in *Rhodobacter sphaeroides*. *Mol Microbiol* **26:** 1083–1096.

Hamblin, P.A., Bourne, N.A., and Armitage, J.P. (1997b) Characterization of the chemotaxis protein CheW from *Rhodobacter sphaeroides* and its effect on the behaviour of *Escherichia coli*. *Mol Microbiol* **24:** 41–51.

Harlow, E., and Lane, D. (1988) *Antibodies: A Laboratory Manual*. New York: Cold Spring Harbor Laboratory Publications.

Harrison, D.M., Skidmore, J., Armitage, J.P., and Maddock, J.R. (1999) Localization and environmental regulation of MCP-like proteins in *Rhodobacter sphaeroides*. *Mol Microbiol* **31:** 885–892.

Hess, J.F., Bourret, R.B., and Simon, M.I. (1988a) Histidine phosphorylation and phosphoryl group transfer in bacterial chemotaxis. *Nature* **336:** 139–143.

Hess, J.F., Oosawa, K., Kaplan, N., and Simon, M.I. (1988b) Phosphorylation of three proteins in the signalling pathway of bacterial chemotaxis. *Cell* **53:** 79–87.

Jeziore-Sassoon, Y., Hamblin, P.A., Bootle, W.C., Poole, P.S., and Armitage, J.P. (1998) Metabolism is required for chemotaxis to sugars in *Rhodobacter sphaeroides*. *Microbiology* **144:** 229–239.

Keen, N.T., Tamaki, S., Kobayashi, D., and Trollinger, D. (1988) Improved broad-host-range plasmids for DNA cloning in gram-negative bacteria. *Gene* **70:** 191–197.

Kim, H., and Farrand, S.K. (1998) Opine catabolic loci from *Agrobacterium* plasmids confer chemotaxis to their cognate substrates. *Mol Plant–Microbe Interact* **11:** 131–143.

Kirby, J.R., Kristich, C.J., Feinberg, S.L., and Ordal, G.W. (1997) Methanol production during chemotaxis to amino acids in *Bacillus subtilis*. *Mol Microbiol* **24:** 869–878.

Klose, K.E., and Mekalanos, J.J. (1998) Differential regulation of multiple flagellins in *Vibrio cholerae*. *J Bacteriol* **180:** 303–316.

Kurdish, I.K., Antonyuk, T.S., and Chuiko, N.V. (2001) Influence of environmental factors on the chemotaxis of *Bradyrhizobium japonicum*. *Microbiology* **70:** 91–95.

Levit, M.N., Liu, Y., and Stock, J.B. (1998) Stimulus response coupling in bacterial chemotaxis: receptor dimers in signalling arrays. *Mol Microbiol* **30:** 459–466.

Maddock, J.R., and Shapiro, L. (1993) Polar location of the chemoreceptor complex in the *Escherichia coli* cell. *Science* **259:** 1717–1723.

Martin, A.C., Wadhams, G.H., and Armitage, J.P. (2001a) The roles of the multiple CheW and CheA homologues in chemotaxis and in chemoreceptor localization in *Rhodobacter sphaeroides*. *Mol Microbiol* **40:** 1261–1272.

Martin, A.C., Wadhams, G.H., Shah, D.S.H., Porter, S.L., Mantotta, J.C., Craig, T.J., *et al.* (2001b) CheR- and CheB-dependent chemosensory adaptation system of *Rhodobacter sphaeroides*. *J Bacteriol* **183:** 7135–7144.

Mowbray, S.L., and Sandgren, M.O. (1998) Chemotaxis receptors: a progress report on structure and function. *J Struct Biol* **124:** 257–275.

Penfold, R.J., and Pemberton, J.M. (1992) An improved suicide vector for construction of chromosomal insertion mutations in bacteria. *Gene* **118:** 145–146.

Porter, S.L., Warren, A.V., Martin, A.C., and Armitage, J.P. (2002) The third chemotaxis locus of *Rhodobacter sphaeroides* is essential for chemotaxis. *Mol Microbiol* in press.

Prentki, P., and Krisch, H.M. (1984) *In vitro* insertional mutagenesis with a selectable DNA fragment. *Gene* **29:** 303–313.

Sambrook, J., and Russell, J.B. (2001) *Molecular Cloning: A Laboratory Manual*. New York: Cold Spring Harbor Laboratory Press.

Schäfer, A., Tauch, A., Jäger, W., Kalinowski, J., Thierbach, G., and Pühler, A. (1994) Small mobilizable multipurpose cloning vectors derived from the *Escherichia coli* plasmids pK18 and pK19 – selection of defined deletions in the chromosome of *Corynebacterium glutamicum*. *Gene* **145:** 69–73.

Shah, D.S.H., Porter, S.L., Martin, A.C., Hamblin, P.A., and Armitage, J.P. (2000) Fine tuning bacterial chemotaxis: analysis of *Rhodobacter sphaeroides* behaviour under aerobic and anaerobic conditions by mutation of the major chemotaxis operons and *cheY* genes. *EMBO J* **19:** 4601–4613.

Sistrom, W.R. (1960) A requirement for sodium in the growth of *Rhodopseudomonas sphaeroides*. *J Gen Microbiol* **22:** 778–785.

Sockett, R.E., Foster, J.C.A., and Armitage, J.P. (1990) Molecular biology of the *Rhodobacter sphaeroides* flagellum. *FEMS Symp* **53:** 473–479.

Springer, W.R., and Koshland, D.E., Jr (1977) Identification of a protein methyltransferase as the *cheR* gene product in the bacterial sensing system. *Proc Natl Acad Sci USA* **74:** 533–537.

Storch, K.F., Rudolph, J., and Oesterhelt, D. (1999) Car: a cytoplasmic sensor responsible for arginine chemotaxis in the archaeon *Halobacterium salinarum*. *EMBO J* **18:** 1146–1158.

Van de Broek, A., Lambrecht, M., and Vanderleyden, J. (1998) Bacterial chemotactic motility is important for initiation of wheat root colonization by *Azospirillum brasilense*. *Microbiology* **144:** 2599–2606.

Wadhams, G.H., Martin, A.C., and Armitage, J.P. (2000) Identification and localization of a methyl-accepting chemotaxis protein in *Rhodobacter sphaeroides*. *Mol Microbiol* **36:** 1222–1233.

Watnick, P.I., Lauriano, C.M., Klose, K.E., Croal, L., and Kolter, R. (2001) The absence of a flagellum leads to altered colony morphology, biofilm development and virulence in *Vibrio cholerae* O139. *Mol Microbiol* **39:** 223–235.

Welch, M., Oosawa, K., Aizawa, S.-I., and Eisenbach, M. (1993) Phosphorylation-dependent binding of a signal molecule to the flagellar switch of bacteria. *Proc Natl Acad Sci USA* **90:** 8787–8791.