Molecular Microbiology (1999) **31**(3), 885–892

Localization and environmental regulation of MCP-like proteins in *Rhodobacter sphaeroides*

David M. Harrison[1,2], Jennifer Skidmore[2], Judith P. Armitage[1] and Janine R. Maddock[2]*

[1]*Microbiology Unit, Department of Biochemistry, University of Oxford, South Parks Road, Oxford OX1 3QU, UK.*

[2]*Department of Biology, University of Michigan, Ann Arbor, MI 48109-1048, USA.*

Summary

Chemotaxis to many compounds by *Rhodobacter sphaeroides* requires transport and at least partial metabolism of the chemoeffector. Previous investigations using phototrophically grown cells have failed to find any homologues of the MCP chemoreceptors identified in *Escherichia coli*. However, using an antibody raised against the highly conserved domain of *E. coli* Tsr, MCP-like proteins were identified in *R. sphaeroides* WS8N. Analysis using Western blotting and immunogold electron microscopy showed that expression of these MCP-like proteins is environmentally regulated and that receptors are targeted to two different cellular locations: the poles of the cells and the cytoplasm. In aerobically grown cells, these proteins were shown by immunoelectron microscopy to localize predominantly to the cell poles and to an electron-dense body in the cytoplasm. Western blot analysis indicated a 17-fold reduction in protein concentration when cells were grown in the light. The number of immunogold particles was also dramatically reduced in anaerobically light-grown cells and their cellular distribution was altered. Fewer receptors localized to the cell poles and more particles randomly distributed within the cell, but the cytoplasmic cluster remained. These trends were more pronounced in cells grown anaerobically under dim light than in those grown anaerobically under bright light, suggesting that expression is controlled by redox state and either light intensity or the extent of photosynthetic membrane synthesis. Recent work on *E. coli* chemosensing suggests that oligomerization of receptors and chemosensory proteins is important for sensory signalling. The data presented here suggest that this oligomerization can occur with cytoplasmic receptors and also provides an explanation for the multiple copies of chemosensory proteins in *R. sphaeroides*.

Received 13 August, 1997; revised 19 October, 1998; accepted 22 October, 1998. *For correspondence. E-mail maddock@biology.lsa.umich.edu; Tel. (+1) 734 936 8068; Fax (+1) 734 647 0884.

Introduction

Bacteria sense the concentrations of many chemoeffectors using methyl-accepting chemotaxis proteins (MCPs). MCPs are typically membrane-bound proteins having two transmembrane domains connecting an amino-terminal periplasmic domain, which senses the concentration of a particular range of chemoeffectors, to a cytoplasmic signalling domain (for a review, see Armitage, 1992; Blair, 1995). This signalling domain appears to be highly conserved across a very wide range of bacterial species (Morgan *et al*., 1993). Methylation of the cytoplasmic domain is important for sensory adaptation and occurs in two regions that flank the signalling domain.

In *Escherichia coli*, the signalling domain of the chemoreceptors forms ternary complexes with two cytoplasmic proteins, CheA and CheW (Gegner *et al*., 1992; Liu *et al*., 1997). Recent data suggest that oligomerization of the ternary complexes may be important for signal sensitivity and gain (Liu *et al*., 1997). Initial studies indicate the critical number of required molecules in a signalling complex may be seven MCP dimers complexing with about four CheW proteins and a CheA dimer (Liu *et al*., 1997). Previous research has found that the chemoreceptor–CheA–CheW complexes predominantly cluster in patches at the cell pole in *E. coli* (Maddock and Shapiro, 1993). The relationship between the chemotaxis oligomers and the polar clusters of MCPs seen by immunoelectron microscopy is currently unknown, but it has been suggested that the clusters represent the large-scale oligomerization of the chemosensory proteins and that changing levels of oligomerization within these 'rafts' of signalling complexes plays an essential role in signal amplification (Bray *et al*., 1998). MCPs are not clustered in strains that lack either CheA or CheW. Similarly, CheA and CheW do not cluster at the poles in strains that lack chemoreceptors. Chemoreceptors have also been shown to cluster and localize to the flagellum-bearing pole of motile swarmer cells of *Caulobacter crescentus* (Alley *et al*., 1992). The amino-terminus of the chemoreceptor is responsible for targeting the protein to the membrane, whereas the carboxyl-terminus of the protein is responsible for its polar localization.

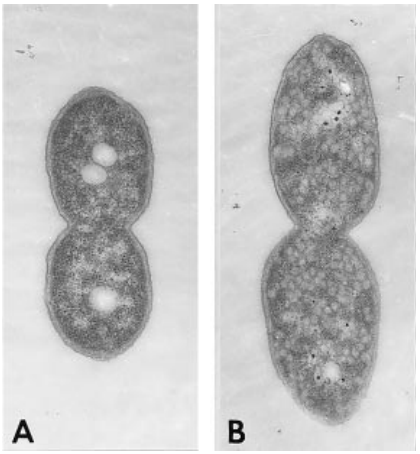

Fig. 1. Thin sections of predivisional cells of *R. sphaeroides* grown aerobically (A) or anaerobically in dim light (B). The intracytoplasmic membranes (ICMs) are clearly visible in cells grown anaerobically in dim light. Magnification is 22 300-fold.

Rhodobacter sphaeroides is a motile, photosynthetic, purple, non-sulphur bacterium capable of growing under a wide range of different conditions including aerobically as a heterotroph and anaerobically as a photoheterotroph, and shows a wide range of behavioural responses to metabolites, oxygen and other terminal acceptors, and to light. *R. sphaeroides* swims by rotating a single subpolar flagellum in the clockwise direction with direction change in occurring when the flagellar motor transiently stops (Armitage and Macnab, 1987). Early work showed that, unlike in *E. coli*, *R. sphaeroides* chemoeffectors had to be transported and at least partially metabolized for a chemotactic signal to occur. In fact, no responses have been seen to non-metabolites (Poole *et al*., 1993; Jacobs *et al*., 1995; Jeziore-Sassoon *et al*., 1998). The use of antibodies raised to the *E. coli* MCPs Tar and Trg in attempts to see methylation of membrane proteins after chemosensory stimulation failed to reveal any MCP-like proteins in *R. sphaeroides* (Sockett *et al*., 1987; Morgan *et al*., 1993). Recently, however, two chemotaxis loci have been identified in *R. sphaeroides* containing multiple homologues of the enteric *cheA*, *cheW*, *cheY* and *cheR* genes (Ward *et al*., 1995a; Hamblin *et al*., 1997). Additionally, these loci contain one or more homologues of *mcp*-like genes (*tpl*, for transducer-like protein). The three *tpl* genes are predicted to encode cytoplasmic MCP signalling proteins because they lack the transmembrane domains (Ward *et al*., 1995b; Armitage and Schmitt, 1997; Hamblin *et al*., 1997). More recently, studies have suggested there may be as many as 12 *mcp*-like genes in *R. sphaeroides*, at least four of which have coding regions for membrane-spanning domains (J. P. Armitage and G. Wadhams, unpublished).

When grown aerobically, the inner membrane of *R. sphaeroides* resembles that of *E. coli*. However, when grown photosynthetically, the inner membrane of *R. sphaeroides* becomes highly invaginated, giving rise to a dense network of intracytoplasmic membranes (Fig. 1) (ICMs) (Chory *et al*., 1984). These harbour the photosynthetic light harvesting (LH) and reaction centre (RC) complexes. Additionally, the cells increase in size during photosynthetic growth compared with aerobic growth (Fig. 1). The synthesis of the photosynthetic complexes is under redox control, triggered by a fall in oxygen concentration (Zelistra-Ryalls *et al*., 1998; Zelistra and Kaplan, 1998). In addition, light intensity controls the extent of LH complex formation, and hence the extent of ICM formation. When grown under dim light, the network of ICMs and associated photosynthetic LH complexes becomes much more extensive to allow the bacterium to harvest more of the available light.

In this study, we have identified and localized *R. sphaeroides* receptor proteins that possess the conserved MCP signalling domain. We have examined these receptors in cells grown anaerobically (photosynthetically) under conditions of high and low light intensity, as well as under aerobic conditions, to answer the following questions.

1. Are the *R. sphaeroides* receptors localized and, if so, are they predominantly clustered at the cell poles as in *E. coli* and *C. crescentus* or around the subpolar flagellum?
2. Given that the ICM could limit the free diffusion of the chemotactic excitatory molecule, CheY-phosphate, does the localization of *R. sphaeroides* receptors vary when cells are grown under different conditions?

Results

Detection of MCP-like proteins in R. sphaeroides

At least one MCP-like protein was detected in *R. sphaeroides* using antibody raised against the highly conserved domain of *E. coli* Tsr. As shown in Fig. 2, this antibody cross-reacts with MCPs in wild-type *E. coli*. There was no cross-reaction with proteins extracted from KO607, an *E. coli* strain in which the four major *mcp* genes are deleted. The antibody also reveals at least two bands of very similar molecular weight (61 kDa) in total cell extracts of *R. sphaeroides* (Fig. 2). It is not known whether the cross-reacting bands are different proteins or reflect different methylation states of one protein. To confirm that the cross-reacting protein(s) was related to chemoreceptors and did not reflect non-specific cross-reactivity of the anti-Tsr antibody, we immunodepleted the Tsr antibody with the conserved signalling domain of Tsr (TsrCSR). Immunodepletion of the Tsr antibody with TsrCSR significantly reduces the immunoreactivity of both *E. coli* and *R. sphaeroides* cell extracts (Fig. 3). Therefore, we have identified at least one *R. sphaeroides* MCP-like or receptor protein antigenically related to the highly conserved *E. coli* signalling domain.

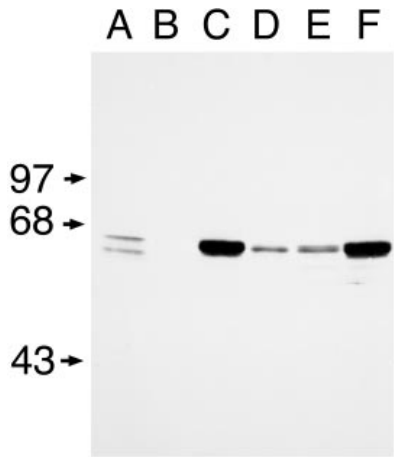

Fig. 2. Western blot using anti-Tsr antibody against total cell extracts of *E. coli* and *R. sphaeroides*. Lanes: (A) *E. coli* RP437; (B) *E. coli* KO607; (C) *R. sphaeroides* WS8N aerobically grown; (D) *R. sphaeroides* WS8N anaerobically grown under bright light; (E) *R. sphaeroides* WS8N anaerobically grown under dim light; (F) *R. sphaeroides* JPA364 (*ΔtlpA*) grown aerobically. An equivalent amount of cells was loaded in each experimental lane. The size of the protein markers is given in kDa.

The immunoreactive protein(s) was produced at a much higher level when the cells were grown under aerobic conditions compared with when they were grown anaerobically with bright or dim light. The relative amount of the *R. sphaeroides* proteins that cross-reacted with the antibody under aerobic and anaerobic conditions was compared by densitometric analysis (Table 1). Because the cross-reacting bands co-migrated under most conditions, the densitometric analysis included all cross-reacting material at 61 kDa. The data show that there was an ≈17-fold increase in the level of cross-reacting protein when the cells were grown aerobically rather than anaerobically under bright light, and an ≈13-fold increase compared with cells grown anaerobically in dim light. This implies that the expression of the genes or genes that encodes the cross-reacting protein(s) is environmentally regulated by oxygen availability and/or light intensity.

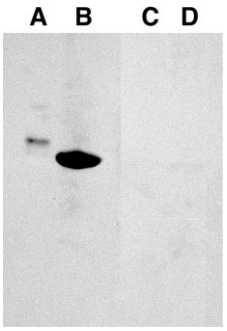

Fig. 3. Immunodepletion of the anti-Tsr antibody. Duplicate samples of total cell extracts of *E. coli* (lanes A and C) and *R. sphaeroides* (lanes B and D) were separated by SDS–PAGE. An equivalent amount of cells was loaded in each experimental lane. After transfer to nitrocellulose, the membrane was cut into strips and treated with either a 1:5000 dilution of anti-Tsr (lanes A and B) or 1:5000 dilution of anti-Tsr preabsorbed to theTsrCSR fragment. The composite image shown is from the same exposure.

The three *R. sphaeroides mcp*-like genes associated with the two chemosensory operons are *tlpA*, *tlpB*, and *tlpC*. Western blot analysis in a *ΔtlpA* strain results in the same intensity and size of cross-reacting material with the anti-Tsr antibody, indicating that TlpA is not the main protein detected by the antibody (Table 1; Fig. 2). TplC does not have a high level of sequence conservation compared with *E. coil* Tsr and is unlikely to be detected by the antibody used. TplB (predicted to be 40 kDa) should not migrate at the position of the cross-reacting protein. Thus, it appears that *R. sphaeroides* possesses at least one protein in addition to the known Tpl proteins with a MCP-like signalling domain and that this additional gene is expressed at a much higher level, particularly under aerobic conditions. Hybridization studies using a probe designed against the signalling domain of Tsr suggest that there may be as many as 12 *mcp*-like genes in *R. sphaeroides* (J. P. Armitage and G. Wadhams, unpublished).

Intracellular location of R. sphaeroides *MCP-like proteins*

The spatial distribution of chemoreceptors in *R. sphaeroides* grown under three different conditions was analysed by immunogold electron microscopy using the antibody raised against *E. coli* Tsr (Fig. 4). The distribution of gold particles in the *ΔtlpA* mutant was similar to that of wild-type cells (data not shown). The number and position of gold particles in 320 transverse sections of predivisional cells grown under each condition was counted and their cellular localization recorded (Table 2). For cells grown under each growth condition, two independent experiments were performed, recording the cellular localization of gold particles in 160 cells in each case.

In aerobically grown cells, the majority of gold particles were located in the membrane (76%). Of these membrane-bound particles, 74% were found in clusters at the poles of the cells. Only 24% of the total gold particles were found in the cytoplasm. The majority of these cytoplasmic gold particles were found associated with the electron-dense body

Table 1. Densitometric analysis of the amount of cross-reacting protein detected in total cell extracts of *R. sphaeroides*, grown under different conditions, with the *E. coli* anti-Tsr antibody.

Growth condition	Relative intensity
WS8N Aerobic	1.0
WS8N Anaerobic: bright light	0.06
WS8N Anaerobic: dim light	0.08
JPA364 Aerobic	0.92

Table 2. Spatial distribution of chemoreceptors detected by immunogold electron microscopy of 320 thin sections of predivisional cells of *R. sphaeroides* WS8N grown aerobically or anaerobically under bright or dim light.

		Anaerobic	
Cellular location	Aerobic	Bright light	Dim light
Total particles	9751	4996	3590
Membrane particles	7459	2863	1641
Polar particles	6952	2481	1361
Polar particles in clusters	5525	1210	546
Lateral membrane particles	507	382	280
Lateral membrane particles in clusters	175	29	4
Total cytoplasmic particles	2292	2133	1949
Cytoplasmic particles associated with electron-dense body	1791	948	1196
Cytoplasmic particles not associated with electron-dense body	501	1185	753

(78%), rather than being randomly distributed throughout the cytoplasm (22%). Most cross sections displayed one or more electron-dense bodies. These bodies were always found towards the centre of the cell and never juxtaposed to the cell membrane. There was no evidence of any membranous structure around the cytoplasmic cluster.

The distribution of gold particles was significantly different in anaerobically grown cells. This is shown in Table 2 and Fig. 4. The total number of gold particles counted in 320 cells was ≈twofold lower when the cells were grown anaerobically with bright light and about threefold lower when the cells were grown using dim light.

In addition, a much lower percentage of gold particles was located in the cytoplasmic membrane when the cells were grown anaerobically: 57% for cells grown using bright light and 46% for cells grown using dim light. In addition, fewer gold particles were found in clusters at the cell poles (Fig. 5), with the size of the polar clusters reduced from 10 to six gold particles, on average, per cell (Table 3). For all growth conditions, only a few of the gold particles were found in the lateral membrane, and when they were found the size of the lateral clusters was much smaller than the size of the polar clusters (Table 3). This is very similar to the situation observed in *E. coli* (Maddock and Shapiro, 1993).

Although the number of gold particles located at the poles was dramatically reduced, approximately the same number of gold particles was located in the cytoplasm for cells grown under aerobic and anaerobic conditions. However, as there were fewer gold particles in total in anaerobic cells, the percentage of total number of particles found in the cytoplasm was consequently higher (43% for cells grown under bright light and 54% for cells grown under dim light). Whether these cytoplasmic particles were associated with ICMs could not be determined because the fixation preparations necessary for immunoelectron microscopy result in poorly preserved membranes. There has been a great deal of research into membrane structure and development in *R. sphaeroides*, and ICMs are not found in aerobically grown cells (Chory *et al*., 1984).

Cells grown under bright light had approximately the same percentage of gold particles associated with the electron-dense body (19%) as aerobically grown cells, whereas more gold particles, as a percentage of total particles, were associated when the cells were grown under dim light (33%). The percentage of cytoplasmic gold particles not associated with this cytoplasmic cluster was much higher in cells grown anaerobically than the percentage of gold particles seen in this location in cells grown aerobically (Table 2). Under anaerobic conditions, the cytoplasm of *R. sphaeroides* becomes packed with ICMs, which contain the photosynthetic light-harvesting and reaction centre complexes (Fig. 1). The amount of ICMs increases in cells grown under low light, as the concentration of LH complexes increases. The gold particles which appeared to be in the cytoplasm, but were not associated with the electron-dense body, may be associated with ICMs rather than being truly cytoplasmic.

Table 3. Size and distribution of clusters. The number of sections with gold particles in clusters and their size when located either in the polar or lateral membrane out of the 320 sections was examined. The relative average size of the clusters is presented ± one standard deviation of the mean for each cell.

Growth condition	Cells with polar clusters	Size of polar clusters	Cells with lateral clusters	Size of lateral clusters
Aerobic	296	10.3 ± 6.1	33	5.3 ± 1.4
Anaerobic: bright light	139	7.0 ± 3.4	7	4.1 ± 0.4
Anaerobic: dim light	77	6.1 ± 2.6	1	4.0 ± 0.0

Discussion

Detection of MCPs

Previous attempts to detect enteric-like MCPs in *R. sphaeroides* were unsuccessful (Sockett *et al*., 1987; Morgan *et al*., 1993). However, using an antibody raised against the highly conserved signalling domain of *E. coli* Tsr (Ames and Parkinson, 1994), we detected a highly specific cross-reaction with *R. sphaeroides* proteins in Western blots (Fig. 2). Because of the specificity of the cross-reacting material, this protein(s) must possess a signalling domain and is almost certainly a receptor protein(s). Although the identity of this protein(s) is unknown, it is likely that it functions in chemotaxis, phototaxis or aerotaxis.

The previous lack of cross-reaction using enteric anti-MCP antibodies was almost certainly the result of a combination of the particular specificity of the antibodies used, and that the antibodies were only used against extracts from cells grown anaerobically in light. We have shown in this study that the expression of *R. sphaeroides* putative MCPs are environmentally regulated: the level of protein increased about 17-fold when the cells were grown aerobically compared with when they were grown anaerobically. The growth conditions used in the previous studies were, therefore, not optimum for a positive cross-reaction with the antibodies used.

The anti-Tsr antibody used in this study did not detect *R. sphaeroides* TlpA, the first MCP-like protein to be sequenced in this organism. The lack of cross-reaction of the anti-Tsr antibody with TlpA may be due to TlpA having a significantly different overall folding pattern as well as its low level of expression (D. M. Harrison and J. P. Armitage, unpublished). TlpA differs substantially from enteric MCPs in its primary structure, having only one putative transmembrane-spanning region and no periplasmic domain. This type of MCP structure is not unique. For example, the archaeon *Halobacterium salinarium* is known to possess three classes of chemoreceptors, all of which have a highly conserved signalling domain but differ in the number of transmembrane domains and whether or not they possess a periplasmic domain (Zhang *et al*., 1996). It will be of interest to determine whether the type of receptor identified with these antibodies is one of the different classes of receptors or more similar to the 'classical' enteric-like MCP(s). Interestingly, the antibody did not appear to recognize the MCP-like aerotaxis receptor Aer, which is still present in the *E. coli* KO607 strain and which also lacks the periplasmic domain. The recent detection of 12 independent genes in *R. sphaeroides* hybridizing with a probe designed to the highly conserved domain of Tsr suggests that the antibody is recognizing one or more of these protein products. A long-term, detailed analysis of these is under way.

Localization of MCP-like proteins

Using immunoelectron microscopy, a *R. sphaeroides* MCP-like protein(s) was shown to cluster predominantly to the cell poles when cells were grown aerobically. Under anaerobic growth conditions, the percentage of total gold particles at the cell poles was greatly reduced and relatively more gold particles were seen in the cytoplasm (Figs 4 and 5; Table 2). Under all growth conditions, cross-reacting protein(s) was also shown to localize to a cytoplasmic electron-dense region. This is the first time that receptors have been shown to aggregate to a specific region within the cytoplasm.

The total number of gold particles was much lower in cells grown anaerobically in the light compared with aerobically grown cells. This parallels the conclusion from the densitometric analysis, though the difference in expression is not as pronounced. When cells were grown aerobically, the total number of gold particles may less accurately reflect the overall number of chemoreceptors in the cell. This is because the large number of gold particles seen in polar clusters may mean that each gold particle may represent a collection of MCPs, with further gold-labelled

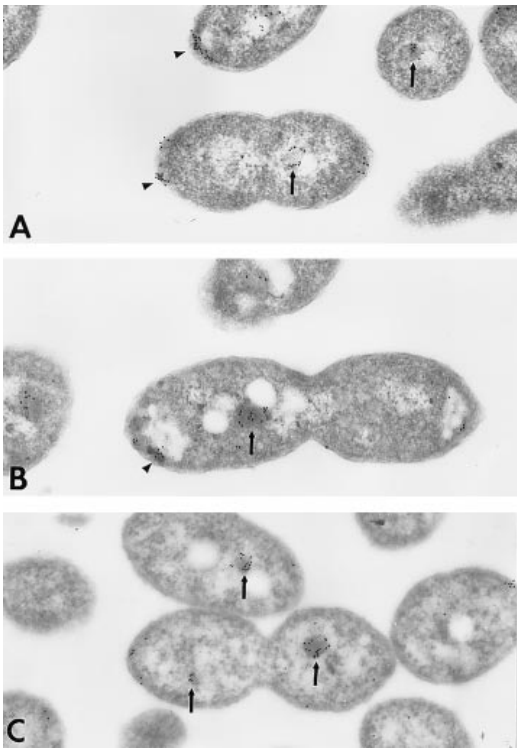

Fig. 4. Cellular distribution of MCP-like proteins in aerobically or anaerobically grown cells. Thin sections of cells grown aerobically (A) or anaerobically under bright (B) or dim (C) light. The position of the MCP-like proteins is detected by the presence of colloidal gold particles. Arrowheads identify MCP-like clusters. The arrows indicate MCP-like proteins associated with the cytoplasmic body. Magnification is 44 600-fold.

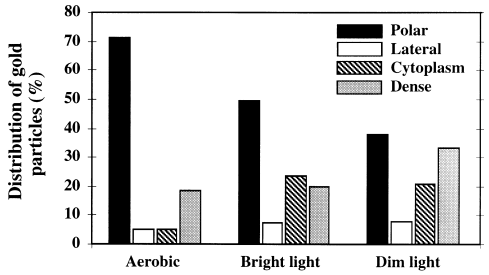

Fig. 5. Distribution of gold particles in predivisional cells of *R. sphaeroides* grown aerobically or anaerobically under bright or dim light. The percentage of gold particles counted in each of four locations is plotted. Polar, gold particles in the membrane at the cell poles; lateral, gold particles in the lateral membrane; dense, gold particles in the cytoplasm associated with the electron-dense body; cytoplasmic, gold particles in the cytoplasm not associated with the electron-dense body.

antibodies being prevented from binding by steric hindrance. The number of gold particles seen at the cell poles and the number of gold particles seen associated with the cytoplasmic electron-dense body were both reduced when the cells were grown anaerobically as opposed to aerobically, implying that both sets of putative MCPs are environmentally regulated by oxygen and/or light intensity. This is the first time that differential regulation of MCP-like proteins has been described and may suggest that different chemosensory pathways may be used under different growth conditions in *R. sphaeroides*. The synthesis of the different components of the photosynthetic apparatus of *R. sphaeroides* is under a complex system of controls involving both redox sensing and light intensity feeding into a pathway of phosphorelay systems (Zelistra-Ryalls *et al*., 1998). It will be interesting to identify whether the expression of the chemosensory system in this bacterium is controlled by the same redox-sensing pathway.

Of the total gold particles, a higher percentage were seen at the cell poles when *R. sphaeroides* was grown aerobically (71%) than when cells were grown anaerobically using bright (50%) or dim (38%) light. A higher percentage of membrane-bound gold particles were clustered at the poles when *R. sphaeroides* was grown aerobically (74%) than anaerobically (42% when using bright light and 33% when using dim light). When grown aerobically, the inner membrane of *R. sphaeroides* resembles that of *E. coli*, when grown under anaerobic conditions the inner membrane of *R. sphaeroides* becomes highly invaginated giving rise to a network of ICMs (Fig. 1). Under anaerobic growth conditions, most of the cytoplasmic gold particles that were not associated with the electron-dense body may have been associated with the ICM. We are unable to visualize membranes clearly with the immunoelectron microscopy preparations and so the association of gold particles with ICMs could not be determined. However, if true, the observed localization pattern supports the model in which the membrane-bound receptors are randomly inserted in the inner membrane and then reach the cell pole by lateral diffusion. When grown anaerobically, the network of ICMs would make this lateral diffusion more difficult, resulting in a lower proportion of chemoreceptors at the cell pole. The reduction in the number of membrane-bound gold particles that are clustered at the pole in anaerobically grown cells may therefore be, in part, due to the dense network of ICMs constraining their net diffusion. The number of polarly clustered membrane-bound gold particles was reduced even further when the amount of ICMs was increased (when cells are grown anaerobically using low light compared with bright light), further supporting this hypothesis.

The identity of the electron-dense bodies to which the receptors localize is not known. This structure has not been reported previously in thin sections of *R. sphaeroides* cells, nor did we unambiguously identify it in our examination of cellular ultrastructure, suggesting that it is most visible under certain fixation and staining conditions. It may be that the structure represents the oligomerized complex of receptors and chemosensory proteins suggested for the polar clusters. Connections between these bodies or between the bodies and the cell membrane were not observed, regardless of the fixation method used. The bodies appear to be isolated entities and may represent the site of synthesis of *R. sphaeroides* MCP-like proteins or, more likely, may be an internal substructure. It is intriguing to speculate that these bodies represent the site of intracellular ternary complex aggregates. If true, this would explain why *R. sphaeroides* has multiple copies of the chemosensory genes. Chemosensory signalling requires the formation of stable complexes between the MCP receptors, CheW, CheA and CheR proteins. It is likely that *R. sphaeroides* responds to both extracellular signals through membrane-bound MCPs and metabolic state via cytoplasmic MCPs, and these are expressed under different conditions. Therefore, one set of chemosensory proteins would be expected to target to the polar MCPs and the other set to the cytoplasm, forming spontaneous aggregates (Gegner *et al*., 1992; Liu *et al*., 1997). Furthermore, it is possible that the type of signalling molecules differ with the alternate cellular locations. For example, phototaxis and aerotaxis receptors, which sense changes in electron transport, may be physically separated from chemotaxis receptors with one type clustered at the cell pole and the other clustered in the electron-dense bodies.

The clustering of the receptor proteins to two distinct cellular locations underscores the importance clustering must play in receptor function (Bray *et al*., 1998). The polar clustering of membrane-bound *E. coli* MCPs probably reflects the need to form clusters with the other chemosensory proteins (Maddock and Shapiro, 1993; Bray *et al*., 1998). The clustering of MCP-like proteins to both the cell

poles and within the cytoplasm also implies that *R. sphaeroides* MCP-like proteins aggregate in order to function optimally. As these cytoplasmic receptors are present under all growth conditions, they may be detecting the concentration of an intracellular metabolite because chemotaxis in *R. sphaeroides* is known to require transport and at least limited metabolism (Ingham and Armitage, 1987; Poole and Armitage, 1989; Jeziore-Sassoon *et al*., 1998). It also suggests that transmembrane signalling is not a prerequisite for the formation of clusters. Work is continuing to identify the MCPs in *R. sphaeroides* and the signals controlling their expression and that of the chemosensory operons.

Experimental procedures

Strains and growth conditions

R. sphaeroides WS8N or JPA 364 (*ΔtlpA*) was either grown aerobically in the dark at 32°C with continuous shaking or anaerobically under conditions of bright light or dim light as previously described, with 7 mM succinate as the carbon source (Harrison *et al*., 1994). *E. coli* strains RP437 (wild-type chemotaxis strain) and KO607 (ΔMCP; Oosawa *et al*., 1988) were grown at 32°C in Luria–Bertani broth. Growth was monitored spectrophotometrically at an OD_{700} for *R. sphaeroides* and an OD_{600} for *E. coli*.

Western blots

One millilitre of mid-log phase motile cells was harvested, then resuspended in 50 μl phosphate-buffered saline (PBS) (140 mM NaCl, 2 mM KCl, 8 mM Na_2HP0_4 and 1.5 mM KH_2P0_4) and 50 μl Laemmli sample buffer (Laemmli, 1970). Samples were frozen at − 20°C and, as needed, were boiled for 5 min and then subjected to electrophoresis on a 10% SDS-polyacrylamide gel against prestained low-molecular-weight markers (Bio-Rad). An equivalent amount of protein was loaded for each experimental lane. The separated proteins were then transferred to nitrocellulose (Scleicher and Schuell) by electrophoresis at 50 mA for 2 h. Filters were blocked overnight at 4°C with 10% dried milk containing 0.1% Tween. Primary antibody (anti-Tsr; Ames and Parkinson, 1994) diluted 1: 5000 in blocking buffer was allowed to adsorb for 1 h at room temperature, then detected using a peroxidase-conjugated goat anti-rabbit secondary antibody (Sigma) and Enhanced Chemiluminescence (Amersham). Quantitation was performed using a LKB Ultra Scan Laser Densitometer.

The TsrCSR fragment was expressed and partially purified from pPA56 in strain RP3098 as described previously (Ames and Parkinson, 1994). Subsequent to ammonium sulphate precipitation, the protein was resuspended to 1 mg ml^{-1} in 25 mM Tris, 1 mM EDTA (pH 8.0). Potassium chloride was added to 200 mM (1:20 dilution) and the protein was heated at 80°C for 35 min and then transferred to an ice-water bath and chilled for 10 min. The sample was transferred to Oak Ridge tubes and centrifuged at 17 000 r.p.m. for 45 min at 4°C. The supernatant was precipitated with 25% ammonium sulphate, pelleted by centrifugation at 15 000 r.p.m. for 30 min at 4°C and then resuspended in 3 ml TE with 2 mM DTT and 10% glycerol. To immunodeplete the anti-Tsr antibodies, 50 μl of the Tsr fragment (approximately 0.1 mg) was incubated with 2 μl anti-Tsr for 30 min at room temperature.

Immunoelectron microscopy

Motile cultures of *R. sphaeroides* were fixed with 3% formaldehyde and 0.1% glutaraldehyde in 30 mM phosphate buffer (pH 7.0) for 1–2 h on ice. The samples were washed three times with phosphate buffer and then treated with 1% sodium metaperiodate for 15 min. After washing once with phosphate buffer, the cells were quenched for 15 min in 50 mM ammonium chloride. The cells were then washed in water, dehydrated in a graded ethanol series and embedded in LR White resin in gelatin capsules at 47°C for 2 days. Sections of 80–100 nm were cut and placed on nickel grids.

The primary antibody (anti-Tsr; Ames and Parkinson, 1994) was diluted 1: 500 in PBST and BSA [140 mM NaCl, 2 mM KCl, 8 mM Na_2HP0_4, 1.5 mM KH_2P0_4, 0.05% Tween 20 and 2% bovine serum albumin (BSA)]. The antibody was adsorbed on ice for 15 min with acetone powders prepared from KO607 (Harlow and Lane, 1988). Nickel grids were submerged in PBST and BSA for 15–30 min and then in the diluted primary antibody in a humidity chamber for 1–2 h. After washing in PBST and then blocking in PBST plus BSA, the grids were then incubated in a 1:30 dilution of 10 nm colloidal gold particles conjugated to goat antibody to rabbit immunoglobulin G (Jackson Immunoresearch) for 1–2 h in a humidity chamber. The grids were washed in water, post-stained with a 1% uranyl acetate solution and examined on a Philips CM10 electron microscope at 60 kV. All of the solutions that were made were passed through a 0.22 μm filter.

All gold particles residing within ≈20 nm of the inner membrane were scored as being membrane associated. This distance reflects the maximum space between the gold conjugate and the membrane-associated epitope. Polar gold particles included those membrane-associated particles at the ends of the cells. A cluster was defined as four gold particles no further than 20 nm apart. Once the core of four gold particles had been identified, all gold particles that were up to 40 nm from any gold particles in the cluster were included. In addition to particles associated with the membrane, in most sections gold particles were seen associated with an electron-dense body in the cytoplasm. These were differentiated from gold particles elsewhere in the cytoplasm in the quantitation (Table 2).

Cellular ultrastructure

When removing samples for immunoelectron microscopy, motile cells of *R. sphaeroides* were also harvested to examine their cellular ultrastructure. Cells were fixed in 2% glutaraldehyde in 0.1 M phosphate buffer (pH 7.0) for 1–2 h on ice. The cells were washed three times in phosphate buffer and then post-fixed in 2% osmium tetraoxide in 0.1 M phosphate buffer (pH 7.0) for 30 min on ice. The cells were washed three times in phosphate buffer and then once in water. Dehydration, infiltration, polymerization of LR White resin and preparation of

grids was performed as described for immunoelectron microscopy. Sections were stained with 1% uranyl acetate and examined as before.

Acknowledgements

We would like to thank Dr J. S. Parkinson for the generous gift of the anti-Tsr antibody, pPA56 and the TsrCSR purification protocol. The authors would also like to thank The Wellcome Trust and the BBSRC for its generous support of this work. The work was also supported in part by a JFRA from the American Cancer Society, GM-55133 from the National Institute of Health and MCB9723749 from the National Science Foundation to J.M. D.M.H. was the recipient of a Wellcome Trust Prize Studentship and travel grants from Balliol College and Oxford University.

References

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

Ames, P., and Parkinson, J.S. (1994) Constituitively signaling fragments of Tsr, the *Escherichia coli* serine chemoreceptor. *J Bacteriol* **176:** 6340–6348.

Armitage, J.P. (1992) Behavioural responses in bacteria. *Ann Rev Physiol* **54:** 683–714.

Armitage, J.P., and Macnab, R.M. (1987) Unidirectional, intermittent rotation of the flagellum of *Rhodobacter sphaeroides*. *J Bacteriol* **169:** 514–518.

Armitage, J.P., and Schmitt, R. (1997) Bacterial chemotaxis: *Rhodobacter sphaeroides* and *Sinorhizobium meliloti* – variations on a theme? *Microbiology* **143:** 3671–3682.

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

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

Chory, J., Donohue, T.J., Varga, A.R., Stahelin, L.A., and Kaplan, S. (1984) Induction of the photosynthetic membranes of *Rhodopseudomonas sphaeroides*: biochemical and morphological studies. *J Bacteriol* **159:** 540–554.

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

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

Harlow, E., and Lane, L. (1988) *Antibodies. A Laboratory Manual*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

Harrison, D.M., Packer, H.L., and Armitage, J.P. (1994) Swimming speed and chemokinetic response of *Rhodobacter sphaeroides* investigated by natural manipulation of the membrane potential. *FEBS Lett* **348:** 37–40.

Ingham, C.J., and Armitage, J.P. (1987) Involvement of transport in *Rhodobacter sphaeroides* chemotaxis. *J Bacteriol* **169:** 5801–5807.

Jacobs, M.H.J., Driessen, A.J.M., and Konings, W.N. (1995) Characterization of a binding-protein dependent glutamate transport system of *Rhodobacter sphaeroides*. *J Bacteriol* **177:** 1812–1816.

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

Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227:** 680–685.

Liu, Y., Levit, M., Lurz, R., Surette, M.G., and Stock, J.B. (1997) Receptor-mediated protein kinase activation and the mechanism of transmembrane signaling in bacterial chemotaxis. *EMBO J* **16:** 7231–7240.

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

Morgan, D.G., Baumgartner, J.W., and Hazelbauer, G.L. (1993) Proteins antigenically related to the methyl-accepting chemotaxis proteins of *Escherichia coli* detected in a wide range of bacterial species. *J Bacteriol* **175:** 133–140.

Oosawa, K., Mutoh, N., and Simon, M.I. (1988) Cloning of the C-terminal cytoplasmic fragment of the Tar protein and effects of the fragment on chemotaxis of *Escherichia coli*. *J Bacteriol* **170:** 2521–2526.

Poole, P.S., and Armitage, J.P. (1989) Role of metabolism in the chemotactic response of *Rhodobacter sphaeroides* to ammonia. *J Bacteriol* **171:** 2900–2902.

Poole, P.S., Smith, M.J., and Armitage, J.P. (1993) Chemotactic signalling in *Rhodobacter sphaeroides* requires metabolism of attractants. *J Bacteriol* **175:** 291–294.

Sockett, R.E., Armitage, J.P., and Evans, M.C.W. (1987) Methylation-independent and methylation dependents chemotaxis in *Rhodobacter sphaeroides* and *Rhodospirillum rubrum*. *J Bacteriol* **169:** 5808–5814.

Ward, M.J., Bell, A.W., Hamblin, P.A., Packer, H.L., and Armitage, J.P. (1995a) Identification of a chemotaxis operon with two *cheY* genes in *Rhodobacter sphaeroides*. *Mol Microbiol* **17:** 357–366.

Ward, M.J., Harrison, D.M., Ebner, M.J., and Armitage, J.P. (1995b) Identification of a methyl-acception chemotaxis protein in *Rhodobacter sphaeroides*. *Mol Microbiol* **18:** 115–121.

Zelistra-Ryalls, H.J., and Kaplan, S. (1998) Role of *fnrL* gene in photosystem gene expression and photosynthetic growth of *Rhodobacter sphaeroides*. *J Bacteriol* **180:** 1496–1503.

Zelistra-Ryalls, H.J., Gomelsky, M., Eraso, J.M., Yeliseev, A., O'Gara, J., and Kaplan, S. (1998) Control of photosystem formation in *Rhodobacter sphaeroides*. *J Bacteriol* **180:** 2801–2809.

Zhang, W., Brooun, A., McCandless, J., Banda, P., and Alam, M. (1996) Signal transduction in the archaeon *Halobacterium salinarium* is processed through three subfamilies of 13 soluble and membrane-bound transducer proteins. *Proc Natl Acad Sci USA* **93:** 4649–4654.