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Polar Localization of $CheA_2$ in *Rhodobacter sphaeroides* Requires Specific Che Homologs

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Rhodobacter sphaeroides **is a motile bacterium that has multiple chemotaxis genes organized predominantly in three major operons (*che*Op_1, *che*Op_2, and *che*Op_3). The chemoreceptor proteins are clustered at two distinct locations, the cell poles and in one or more cytoplasmic clusters. One intriguing possibility is that the physically distinct chemoreceptor clusters are each composed of a defined subset of specific chemotaxis proteins, including the chemoreceptors themselves plus specific CheW and CheA proteins. Here we report the subcellular localization of one such protein, $CheA_2$, under aerobic and photoheterotrophic growth conditions. $CheA_2$ is predominantly clustered and localized at the cell poles under both growth conditions. Furthermore, its localization is dependent upon one or more genes in *che*Op_2 but not those of *che*Op_1 or *che*Op_3. In *E. coli*, the polar localization of CheA depends upon CheW. The *R. sphaeroides che*Op_2 contains two *cheW* genes. Interestingly, $CheW_2$ is required under both aerobic and photoheterotrophic conditions, whereas $CheW_3$ is not required under aerobic conditions but appears to play a modest role under photoheterotrophic conditions. This suggests that *R. sphaeroides* contains at least two distinct chemotaxis complexes, possibly composed of proteins dedicated for each subcellular location. Furthermore, the composition of these spatially distinct complexes may change under different growth conditions.**

It has been well documented that the localization of some bacterial proteins to specific regions in the cell is essential for their correct functioning. Examples of these include proteins involved in *Caulobacter crescentus* development, *Bacillus subtilis* sporulation, and *Escherichia coli* cell division (for a review, see reference 20). The chemotaxis pathway, which allows bacteria to move in a favorable direction, also has components that are specifically localized within the cell (14). In enteric bacteria, chemotaxis is mediated by a classical two-component signal transduction pathway (for reviews, see references 2, 4, and 26). The protein kinase CheA is phosphorylated on a conserved histidine residue due to a change in the signaling state of a *trans*-membrane chemoreceptor (*trans*-MCP). The phosphoryl group is transferred to the response regulator CheY. CheY-P binds to the flagellar switch protein FliM, causing the direction of flagellar rotation to change from counterclockwise to clockwise and ultimately resulting in a change of swimming direction. Adaptation to stable chemoeffector concentrations is accomplished by modification of the chemoreceptors using two enzymes working antagonistically. The constitutively active methyltransferase CheR adds methyl groups to specific glutamate residues of the *trans*-MCPs, thereby increasing the activity of CheA (1). CheB, when phosphorylated by CheA, removes these methyl groups, thus decreasing CheA activity (12).

In all bacteria and archaea examined thus far, the chemoreceptor complexes are clustered (6, 9, 13, 14). Although the function of clustering is currently unknown, one possibility is that clustering may allow cooperative interactions between receptors, facilitating signal generation, signal amplification and/or adaptation (3, 5, 10, 11, 21). In *E. coli*, the kinase CheA and the scaffolding protein CheW are also specifically localized to the cell poles (14), where they may form higher order signaling arrays with the chemoreceptors (3, 10, 11, 21).

Many motile bacteria sense and respond to environmental changes by employing variations of the *E. coli* paradigm. The α-subgroup bacterium *Rhodobacter sphaeroides* is a metabolically diverse species that has multiple homologs of the *E. coli* signaling proteins encoded in three operons and at other unlinked loci (www.jgi.doe.gov/JGI_microbial/html/rhodobacter). *che*Op_1 contains $cheY_1$, $cheA_1$, $cheW_1$, $cheR_1$, and $cheY_2$. *che*Op_2 contains $cheY_3$, $cheA_2$, $cheW_2$, $cheW_3$, $cheR_2$, $cheB_1$, and *tlpC*. *che*Op_3 contains $cheA_4$, $cheR_3$, $cheB_2$, $cheW_4$, *slp*, *tlpT*, $cheY_6$, and $cheA_3$. In total, there are four CheAs, four CheWs, six CheYs, three CheRs, and two CheBs. In addition, there are a CheBRA fusion protein (encoded at a separate locus) and 13 chemoreceptors. Nine chemoreceptors are membrane-spanning and four are cytoplasmic, known as transducer-like proteins (Tlps). Immunoelectron microscopy using an antibody against the highly conserved domain of *trans*-MCPs showed that receptor proteins are clustered at both the cell poles and in the cytoplasm (9). Specific green fluorescent protein (GFP) fusions showed that the *trans*-membrane receptor, McpG, was located at the poles (24) and the putative cytoplasmic receptor, TlpC, formed discrete foci within the cytoplasm of the cell (25). Defining the distribution of these Che proteins in the cell and determining their interplay is critical for truly understanding chemotaxis in *R. sphaeroides*.

$CheA_2$ (encoded in *che*Op_2) is essential for aerotaxis, phototaxis, and chemotaxis to all compounds tested and for the localization of McpG to the cell pole (15). Deletion of $CheA_2$

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TABLE 1. Strains used in this study

Strain	Characteristic	Source or reference
WS8	Wild-type *R. sphaeroides*	Gift from W. Sistrom
WS8N	Spontaneous nalidixic acid-resistant mutant of WS8	23
JPA117	Δ*che*Op_1 derivative of WS8N	7
JPA1301	Δ*che*Op_3 derivative of WS8N	17
JPA1340	Δ*che*BRA derivative of WS8N	Gift from S. L. Porter
JPA1349	Δ *che*BRA *cheY7* derivative of WS8N	Gift from S. L. Porter
JPA211	Δ*che*A_2 derivative of WS8N	7
JPA470	Δ*tlpC* derivative of WS8N	25
JPA514	Δ*che*W_2 derivative of WS8N	15
JPA517	Δ*che*B_1 derivative of WS8N	16
JPA527	Δ*che*W_3 derivative of WS8N	15
JPA531	WS8N containing an Ω cartidge interrupting transcription and translation of *mcpG*	24
JPA565	Δ*che*R_2 derivative of WS8N	16

results in some, but not total, delocalization of TlpC (25). In contrast, deletion of *che*A_1 has only minor effects on chemosensing and is not required for either the localization of McpG (15) or TlpC (25). In this study we examined the subcellular localization of $CheA_2$ in *R. sphaeroides* and systematically investigated the requirement for other signaling proteins in that localization.

MATERIALS AND METHODS

Strains and growth conditions. Strains of *R. sphaeroides* (Table 1) were grown in succinate medium (22) containing nalidixic acid (25 μg/ml) at 30°C either aerobically with shaking in the dark or anaerobically with illumination at 50 μmol m^{-2} s^{-1}.

Antibody production. Purified His-tagged $CheA_2$ was made as described previously (17). Rabbit polyclonal antibodies to His-tagged $CheA_2$ (Eurogentec) detect both His-tagged $CheA_2$ protein and a protein from *R. sphaeroides* WS8N extracts of the expected molecular mass (69.4 kDa). This immunoreactive protein was absent from the Δ*che*A_2 control strain (JPA211). The antiserum was immunodepleted before use with acetone powders prepared from JPA211 by standard methods (8).

Electron microscopy. Motile cultures of *R. sphaeroides* were fixed, embedded in LR-White resin, sectioned and placed on nickel grids as described previously (9). Immunoelectron microscopy was performed using a 1:500 dilution of primary antibody and a 1:30 dilution of secondary antibody (12-nm-diameter colloidal gold particles conjugated to goat antibody to rabbit immunoglobulin G; Jackson Immunoresearch) as described previously (9).

The intracellular positions of all gold particles in longitudinal sections of predivisional cells were recorded. Gold particles within 20 nm of the membrane were scored as being membrane associated. These were further subdivided into those along the lateral membrane (lateral) and those associated with the polar membrane (polar). We also tracked the colocalization (clustering) of gold particles. For this study, a cluster was defined as three or more gold particles each located no more than 20 nm from its neighbor, together with any outlying particles that were no more than 40 nm from the core cluster. Statistical analysis was performed using the χ^2 test.

Immunoblotting. Because the packing of proteins can influence the number of gold particles, using immunoelectron microscopy, $CheA_2$ levels were monitored by immunoblotting. Motile cells (1 ml, optical density at 700 nm = 0.6) were harvested and resuspended in 100 μl of sample buffer (0.05 M Tris-Cl [pH 6.8], 10% glycerol, 1% sodium dodecyl sulfate, 0.05 M dithiothreitol, 0.01% bromophenol blue), and 10 μl was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (12% polyacrylamide) and electroblotted by standard methods (18). The membranes were blocked in 5% dried milk, incubated for 1 h in preabsorbed sera diluted 1/2,000 in phosphate-buffered saline (PBS) containing 1% dried milk, and washed extensively with PBS. The membrane was then blocked in PBS containing 0.2% Tween 20, incubated for 1 h with a 1/1,000 dilution of anti-rabbit horseradish peroxidase conjugate (Dako), and washed, and bands were detected by enhanced chemiluminescence (Amersham Pharmacia Biotech).

To determine the copy number of $CheA_2$ in the cell, quantitative immunoblots of dilution series of WS8N extracts (prepared as above) and $CheA_2$ protein were performed. The mean of results frp, three independent experiments was taken.

RESULTS

Localization of $CheA_2$. To determine the localization of $CheA_2$ in aerobically grown cells, immunoelectron microscopy experiments were performed using $CheA_2$ antibody on ultrathin sections of wild-type cells. Of the gold particles, 78% were associated with the membrane, and 86% of these were polar (67% of the total particles) (Fig. 1; Table 2). Analysis of the polar signal revealed that typically, more gold particles were seen at one pole than at the other (71% of cells had at least two more particles at one pole than at the other), perhaps reflecting a bias for the localization of $CheA_2$ to the older pole. Polar $CheA_2$ was moderately clustered (52%). There were on average 8.6 gold particles per cell section, with very little noise evident (0.4 particles per cell in the Δ*che*A_2 control strain, JPA211). Therefore, the antibody does not cross-react with the other CheA species. Some $CheA_2$ is found in the cytoplasm (1.9 cytoplasmic particles per cell compared to 0.2 in JPA211), but no specific positioning of the gold particles was observed and they were not clustered. Thus, the majority of $CheA_2$ is localized to the poles of the *R. sphaeroides* cell.

Dependence of $CheA_2$ localization on components of *che*Op_1 and *che*Op_3, on CheBRA, and on McpG under aerobic conditions. To determine whether Che proteins encoded by *che*Op_1, *che*Op_3, or *cheBRA* were required for the polar localization of $CheA_2$, immunoelectron microscopy was performed on sections of strains with deletions of *che*Op_1 (JPA117), *che*Op_3 (JPA1301) and *cheBRA* (JPA1340). Since both $CheA_2$ and $CheW_2$ are required for the localization of McpG under aerobic conditions (15), we also examined the requirement for McpG in $CheA_2$ localization in a strain in which *mcpG* had been insertionally inactivated by an Ω cartridge (JPA531) (24). The data obtained from all these strains were very similar to those obtained from wild-type cells (data not shown). Therefore, none of the components of *che*Op_1 or *che*Op_3 (which include genes encoding $CheA_1$, $CheA_3$, $CheA_4$, $CheW_1$, and $CheW_4$), *cheBRA*, or *mcpG* are required for $CheA_2$ localization.

Dependence of $CheA_2$ localization on components of *che*Op_2 under aerobic conditions. Proteins encoded within the same operon as $CheA_2$ were examined, individually, for any role in $CheA_2$ localization. The pattern of $CheA_2$ localization in strains from which *che*W_3 (JPA527), *che*R_2 (JPA565), *che*B_1 (JPA517), and *tlpC* (JPA470) were deleted was not significantly different from that of the wild-type strain ($P > 0.05$)

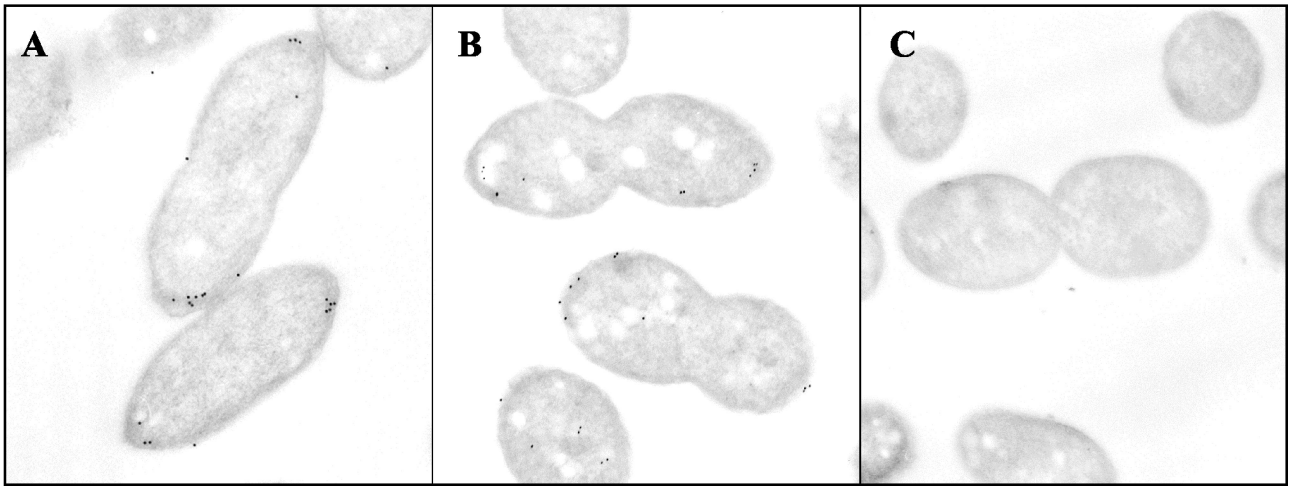

FIG. 1. $CheA_2$ localizes predominantly to the cell poles in *R. sphaeroides* cells. Ultrathin sections of wild-type cells grown aerobically (A) and photoheterotrophically (B) were incubated with an antibody to $CheA_2$ and detected with an anti-rabbit colloidal gold conjugate. Few gold particles are present in JPA211, a strain lacking *cheA*$_2$ (C). The micrographs show the location of $CheA_2$ as detected by immunoelectron microscopy experiments using an antibody to $CheA_2$.

(Table 2). In the absence of $CheW_2$ (JPA514), however, the number of polar gold particles decreased dramatically (1.0 and 5.8 polar membrane particles in cells lacking *cheW*$_2$ and in the wild type, respectively) (Table 2; Fig. 2). Only 15% of the signal was polar (compared to 67% for the wild type) and very few polar particles were in clusters (5%). There was a concomitant 2.4-fold increase in the number of cytoplasmic particles and an apparent 1.5-fold increase in the number of lateral membrane particles. Despite there being an increase in the number of cytoplasmic and lateral particles, they were not clustered. These data show that $CheW_2$ is required for normal $CheA_2$ polar localization and clustering.

Dependence of $CheA_2$ localization under photoheterotrophic conditions. We have previously shown that $CheW_3$ has a more pronounced role in chemotaxis and in McpG localization under photoheterotrophic conditions than under aerobic conditions (15). Therefore, we addressed whether $CheW_3$ had a role in the localization of $CheA_2$ in cells grown photoheterotrophically.

The pattern of $CheA_2$ localization in photoheterotrophically grown wild-type cells was very similar ($P > 0.05$) to that observed in aerobically grown cells (Fig. 1). The amount of signal, however, was approximately twofold lower (3.9 and 8.6 spots per cell section under photoheterotrophic and aerobic conditions, respectively). When grown photoheterotrophically, 75% of the gold particles were associated with the membrane and 88% of these were polar (Table 3). Clustering of the polar particles was lower than in aerobically grown cells (27 and 52%, respectively) but may be a consequence of the lower signal observed (2.6 and 5.8 polar membrane particles under photoheterotrophic and aerobic conditions, respectively).

In the absence of $CheW_2$, under photoheterotrophic conditions, the percentage of particles that were polar decreased from 66 to 15% ($P < 0.001$) (Table 3), consistent with a critical requirement for $CheW_2$ under these conditions. The 4.3-fold decrease in the average number of polar particles was accompanied by a 2.8-fold increase in the cytoplasmic signal and a 2.1-fold increase in the lateral membrane signal. Interestingly, in the absence of $CheW_3$, the percentage of polar particles decreased to 56%, a much smaller but nevertheless statistically significant reduction ($P < 0.05$) (Table 3). In addition, a concomitant increase in the number of cytoplasmic and lateral particles was observed in cells lacking $CheW_3$ (Fig. 2). These data demonstrate that $CheW_2$ and, to a lesser extent, $CheW_3$ are required for the normal localization of $CheA_2$ under photoheterotrophic conditions.

Expression of $CheA_2$ is not affected by the deletion of other *che* genes. Quantitative immunoblots of $CheA_2$ in the appropriate deletion strains were performed to ensure that any observed differences in the pattern of $CheA_2$ localization was not

TABLE 2. Spatial distribution of $CheA_2$ in aerobically grown wild-type strains and deletion mutants[a]

Strain	No. polar	Polar in clusters	No. lateral	No. cytoplasmic	Cytoplasmic in clusters	No. of particles/section	No. of polar particles/section	No. of cytoplasmic particles/section
WS8N	930	482	146	304	9	8.6	5.8	1.9
Δ*cheA*$_2$	33	10	14	33	0	0.4	0.2	0.2
Δ*cheR*$_2$	838	364	100	186	3	7.0	5.2	1.2
Δ*cheB*$_1$	817	321	90	171	6	6.7	5.1	1.1
Δ*tlpC*	781	320	89	199	0	6.7	4.9	1.2
Δ*cheW*$_2$	175	9	222	743	0	7.0	1.0	4.6
Δ*cheW*$_3$	651	259	171	258	12	6.8	4.1	1.6

[a] For each strain 160 longitudinal sections were scored.

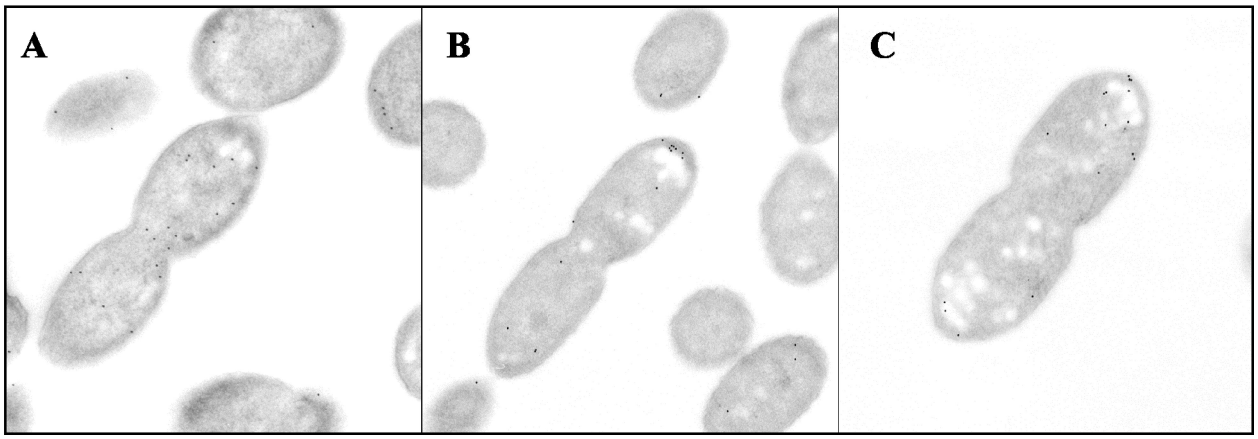

FIG. 2. The localization of $CheA_2$ in aerobically grown cells is significantly affected by a deletion of *cheW*$_2$ (A), only slightly affected by the deletion of *cheW*$_3$ under aerobic conditions (B), and moderately affected by the deletion of *cheW*$_3$ under photoheterotrophic conditions (C). The micrographs show the location of $CheA_2$ as detected by immunoelectron microscopy experiments using an antibody to $CheA_2$.

due to changes in $CheA_2$ expression levels. In aerobic cells the level of $CheA_2$ remained constant in all the strains used in this study, with the exception of JPA211, the negative control (Fig. 3). Under photoheterotrophic conditions, the total level of $CheA_2$ was reduced, but at a consistent level, in all strains except the negative control strain. Therefore, the $CheA_2$ localization pattern in the deletion strains was not influenced by $CheA_2$ levels under either environmental condition.

Quantitative immunoblots using known amounts of purified $CheA_2$ protein showed that the number of copies of $CheA_2$ in *R. sphaeroides* was approximately 7,000 and 1,000 per cell under aerobic and photoheterotrophic conditions, respectively. This is consistent with the lower signal observed in photoheterotrophic cells by immunoelectron microscopy.

DISCUSSION

$CheA_2$ is the most highly expressed CheA in *R. sphaeroides* (19) and is essential for all measured responses under both aerobic and photoheterotrophic conditions (15). Here we show that $CheA_2$ is clustered at the cell pole. It is now well established that the chemoreceptors form clusters or signaling complexes at the cell poles of a variety of bacterial species (6, 14). In *R. sphaeroides*, however, receptors are clustered at the cell poles and in the cytoplasm (9). We have previously shown using GFP fusions that McpG predominately localizes to the poles (24) whereas TlpC localizes to cytoplasmic foci. It seems probable that $CheA_2$ forms part of a polar signaling complex that includes McpG.

$CheA_2$ was only moderately clustered at the cell pole (52% of polar membrane particles are clustered). One intriguing possibility is that $CheA_1$, $CheA_3$, and/or $CheA_4$ is in the same signaling array and, therefore, reduces the density of $CheA_2$ within this array. It remains to be determined, however, if these other CheA homologs localize to the cell poles or to the cytoplasmic cluster. Interestingly, the pattern of localization of $CheA_2$ was the same in cells grown aerobically and photoheterotrophically even though the copy number of $CheA_2$ protein was sevenfold higher in aerobically grown cells, suggesting no significant redistribution of $CheA_2$ under these different environmental conditions.

Deletion of *cheW*$_2$ resulted in a major reduction in the polarity and clustering of $CheA_2$ in both aerobic and photoheterotrophic cells. In the absence of $CheW_2$, $CheA_2$ was predominantly in the cytoplasm although some $CheA_2$ was also associated with the lateral membrane (Fig. 2). These data are consistent with those from *E. coli*, where CheW (the only CheW in *E. coli*) was found to be required for the polarity of both the chemoreceptors and CheA (14). Deletion of *cheW*$_3$ resulted in a modest reduction in $CheA_2$ polar localization only in photoheterotrophically grown cells, suggesting that $CheW_2$ is absolutely essential for $CheA_2$ localization while $CheW_3$ may only be required for optimal $CheA_2$ localization in photoheterotrophic cells. These results are consistent with previously published data that described the localization of McpG (15). There is evidence that the chemoreceptors of *R. sphaeroides* are differentially expressed according to the environmental condition (9); therefore, the receptors that are more highly expressed under photoheterotrophic conditions may require both $CheW_2$ and $CheW_3$ for optimal packing in the array.

TABLE 3. Spatial distribution of $CheA_2$ in photoheterorophically grown wild-type strains and deletion mutants[a]

Strain	No. polar	Polar in clusters	No. lateral	No. cytoplasmic	Cytoplasmic in clusters	No. of particles/section	No. of polar particles/section	No. of cytoplasmic particles/section
WS8N	415	113	59	154	3	3.9	2.6	1.0
Δ*cheA*$_2$	105	0	14	50	0	0.5	0.1	0.3
Δ*cheW*$_2$	103	0	123	444	23	4.2	0.6	2.8
Δ*cheW*$_3$	368	188	104	188	16	4.1	2.3	1.2

[a] For each strain 160 longitudinal sections were scored.

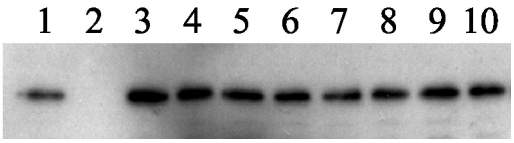

FIG. 3. Expression of $CheA_2$ is not affected by the deletion of other Che proteins. Shown is a quantitative immunoblot of $CheA_2$ in the wild type (lane 1) and in all the appropriate deletion strains under aerobic conditions: Δ*tlpC* (lane 3), Δ$cheB_1$ (lane 4), Δ$cheR_2$ (lane 5), Δ$cheW_3$ (lane 6), Δ$cheW_2$ (lane 7), Δ*cheBRA* (lane 8), Δ*che*Op_3 (lane 9), and Δ*che*Op_1 (lane 10). There is no band in the Δ$cheA_2$ negative control (lane 2).

Deletion of *mcpG* does not affect $CheA_2$ localization. The absence of a single, albeit highly expressed chemoreceptor, is insufficient to disorder the polar $CheA_2$ cluster. In *E. coli*, CheA and CheW polar clustering is disrupted in the absence of all of the chemoreceptors (14); however, expression of one chemoreceptor is sufficient to sequester both CheA and CheW to the cell poles (13, 14). Thus, CheA is targeted to the pole provided that at least one chemoreceptor is present (14). Given that *R. sphaeroides* has eight additional predicted membrane receptors, the localization of $CheA_2$ in the absence of McpG is not surprising.

In *R. sphaeroides*, there appear to be two discrete regions in the cell that are absolutely required for chemotactic response: the cell poles and the cytoplasmic foci. Whether there is communication between the two loci to produce a balanced response at the single flagellar motor is the subject of ongoing experimentation. In the absence of $CheA_2$ there was a small, yet consistent, delocalization of TlpC-GFP fluorescence. It seems likely that deletion of $cheA_2$ affects the stoichiometry of other CheA, CheW, and receptor proteins, resulting in a modest TlpC delocalization as $CheA_2$ was not found associated with the cytoplasmic clusters and therefore probably does not have a major role in signaling from the cytoplasmic receptors.

These data are consistent with a recently proposed model (15) which suggests that there are polar signaling arrays containing up to nine chemoreceptor homologs, $CheA_2$, $CheW_2$, and, under photoheterotrophic conditions when different receptors may be expressed, $CheW_3$. Similarly, the cytoplasmic clusters may be composed of up to four soluble receptors, including TlpC (25) and other dedicated CheA and CheW proteins. Interestingly, it is also likely that these spatially distinct complexes also share one or more components and may be more dynamic, as evidenced by the dual role that $CheW_3$ plays in signaling from both polar and cytoplasmic clusters (15, 25).

We have shown that genes in both *che*Op_2 and *che*Op_3 are essential for chemotaxis and that proteins encoded by the two operons are found in two locations, the cell poles and a cytoplasmic cluster. Disruption of either the polar or cytoplasmic cluster appears to result in the loss of taxis (15). The data presented here show that $CheA_2$, itself essential for all tactic responses (15), is found only in the polar clusters and not in the cytoplasmic cluster. This suggests that essential chemosensory proteins may be differentially targeted to different cellular locations.

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