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Cell biology of bacterial sensory modules

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1. ABSTRACT

Despite their small size, bacterial cells possess very efficient sensory apparatus that allow them to perceive and respond to the external environment with cell movement. In enteric bacteria, these apparatus are complex lattices of different chemoreceptors working in concert and forming clusters positioned at the cell poles. Since the study of chemotaxis has been expanded to other bacterial species, examples of chemosensory systems regulating functions different than taxis have been described and chemoreceptors localizing in ways divergent from the enteric paradigm have been visualized. The scope of this review is to revise and summarize the architecture of different bacterial chemoreceptors. Then, hypotheses will be proposed on how chemoreceptor distribution in cells is coupled to specific functions and life styles in well-characterized bacterial model systems, such as *Escherichia coli*, *Rhodobacter sphaeroides*, *Caulobacter crescentus* and *Myxococcus xanthus*.

2. INTRODUCTION

The sensing and response to certain molecules by moving cells is a behavior that is widespread in prokaryotes and eukaryotes and linked to several important functions. For example, a fundamental feature of any immune response is the movement of leucocytes from one site in the body to another, to provide effector functions. Cell movement is oriented in relation to ligand gradients. In protozoa, algae and bacteria, chemotaxis is employed to reach nutrients and exert virulence processes. Chemosensing has also been associated with the formation of bacterial communities and specialized cell forms like spores or cysts (8).

Howard Berg (Nature 1982) described chemotaxis following observations of the three-dimensional movement of *E. coli* cells in the absence or presence of a gradient of chemicals as follow: “In the absence of a stimulus (i.e. no attractant or repellent present,

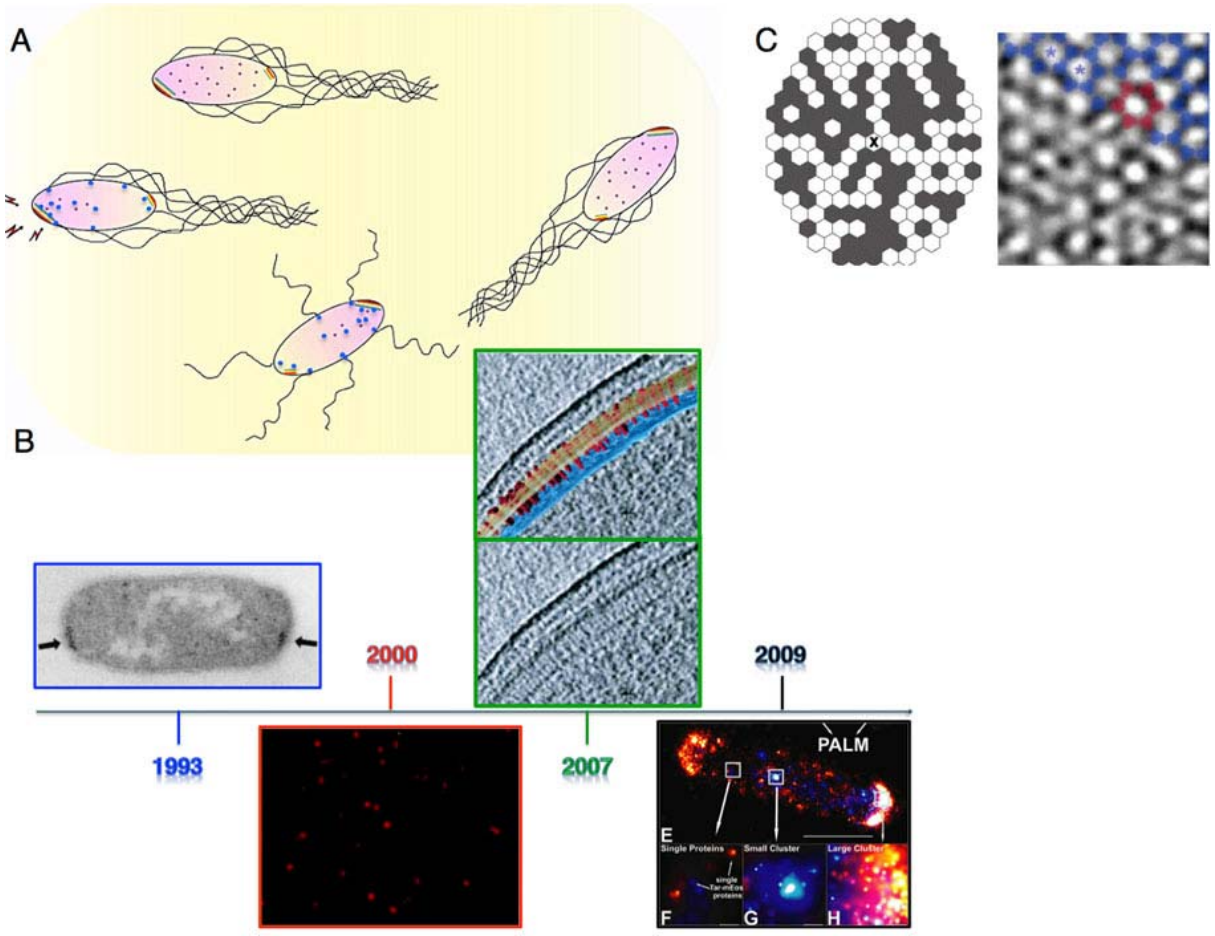

Figure 1. (A) Schematic representation of an *E. coli* cell swimming in a direction and responding to an increasing concentration of a chemical with a tumbling and a consequent change of direction. Tumbling is stimulated by a temporary accumulation of CheY-P molecules (blue; unphosphorylated CheY are represented in purple), which communicate to the perithrichious flagellar motors to switch the sense of rotation. (B) (Left panel) Representation of the model proposed by Bray *et al.*, where hexagons represent individual receptors in the high-active (grey) or low-active state (white). The receptor in the center (cross) is bound to a ligand and will transfer its low-active conformation to the surrounding receptors, thus amplifying the initial signals of several folds. This figure was reproduced from Bray *et al.*, Nature, 393(85-88), with permission of Nature Publishing Group. (Right panel) Face-up view of a chemoreceptor lattice resolved by cryo-electron tomography. This figure was reproduced from Briegel et a., PNAS, 106(17181-6) with permission of PNAS. (C) Localization of Tar by different techniques. From the left: immunogold electron-microscopy, fluorescence microscopy, cryo-electron tomography and PALM. This figure was reproduced with permission of PNAS (14).

or else constant, uniform concentration - no gradient) a bacterium such as *E. coli* or *S. typhimurium* swims in a smooth, straight line for a number of seconds - a "run" then it thrashes around for a fraction of a second – a "tumble" (or abruptly changes its direction - a "twiddle"); and then it again swims in a straight line, but in a new, randomly chosen direction. (A tumble is probably a series of very brief runs and twiddles.)" (Figure 1A) (69). The tactic response to chemicals is a phenomenon largely more complex and finely regulated than a simple stimulation. In a simple stimulation response, a one or a two-component system is activated by a signal, then a histidine kinase domain autophosphorylates and transfers the phosphoryl group to a response regulator domain, which, in turn, generates the final response. Thus, a simple response depends on a system that is either on or off. Chemotactic responses are, instead, mediated by two-component systems that have evolved, modified and enriched of accessory components and functions that provided further degrees of regulation to the "on or off" status. Maximum sensitivity, quick resetting of the system and amplification of the signal are some of the several features that were acquired during the evolution of two-component systems into more complex chemotaxis pathways. Interestingly, over the past years more chemosensory systems have been identified that do not regulate taxis, but rather behavioral responses to the environment. Bacteria possessing these divergent chemosensory systems show highly complex physiology, metabolism and behaviors, when compared to the enteric model.

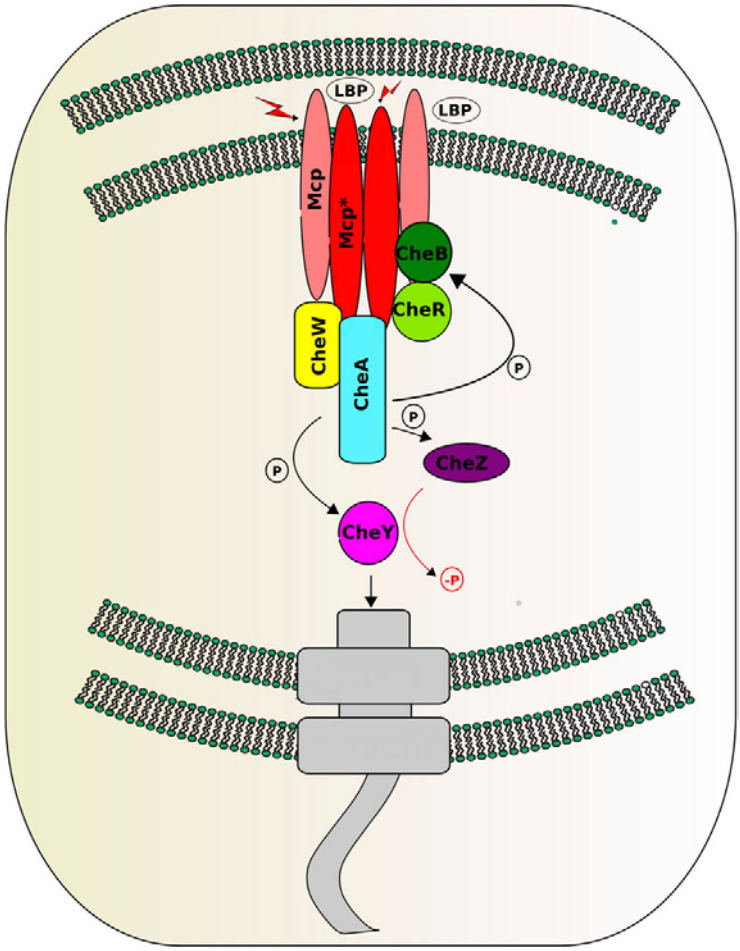

Figure 2. Schematic representation of the *E. coli* chemosensory system. Trasmembrane receptors perceive signals directly (red flash) or via ligand binding proteins (LBP). Mcps are divided in high-represented (Mcp*, dark red) and low-represented Mcps (light red). CheR proteins (light green) bind the C-terminal NWETF pentapeptide only present on the high-represented Mcps. The flagellar motor is represented in grey.

In enterics, chemoreceptors are organized in one or two major polar clusters, visible by standard microscopy, but also small lateral clusters (see below) (15, 21, 45, 73, 83, 101) (Figure 1B). Clusters contain chemoreceptors with different specificities (Figure 2) and forming, altogether, an array that is able to sense a mixture of signals and translate them in regulated cell movement (3, 40, 59, 76, 78). Relatively recent results have shown that the architecture of bacterial chemoreceptors can largely vary among different bacterial species and it is likely associated with life styles, behaviors and functions characterizing these species (Table 1). For example, the distribution of chemoreceptors becomes complex in *Rhodobacter sphaeroides*, a bacterium with a high metabolic flexibility and displaying taxis towards a wide range of compounds. *Rhodobacter* is the first bacterium described for having multiple chemosensory systems (23, 62, 96). One of the three *R. sphaeroides* chemotaxis systems localizes, similarly to enterics, in one polar cluster and another one forms a cytoplasmic cluster positioned at the center of cells (85, 92, 94). Even higher complexity is reached in the gliding social bacterium *Myxococcus xanthus*, where genetic analyses revealed the presence of eight chemotaxis-like systems and a total of 21 chemoreceptors localizing in singular patterns (48)(104).

It has been described that, in most cases, the number of one and two component systems present in a bacterial genome directly relates to the complexity of the life cycle of the given bacterium (89). The same must be true for chemoreceptors and chemotaxis systems: a high number of chemoreceptors and chemosensory systems arranged in different locations in cells might reflect complex behaviors. In this review, we will propose hypotheses on how different localization patterns are coupled with behaviors and life styles of different bacterial species.

3. POLAR CLUSTERS: THE ENTERIC PARADIGM

3.1. *E. coli* Che system

E. coli is the bacterial species where chemotaxis has been mostly studied and is, therefore, the

Table 1. Chemosensory and motility modules in different bacterial species

	Number of *che* operon	Number of transmembrane Mcp	Number of cytoplasmic Mcp	Mcp cluster position	Motility systems
Escherichia coli	1	5	0	Two large polar clusters and several small lateral clusters	Peritrichous flagella
Caulobacter crescentus	2	12	6	One cluster at flagellated pole	One flagellum
Rhodobacter spheroides	3	9	4	One polar cluster and one cytoplasmic cluster	One flagellum
Myxococcus xanthus	8	19	2	Multiple distributer clusters	Polar type IV pili and distributed focal adhesion complexes

best characterized to date. For this reason, investigators established paradigms based on the *E. coli* system that have been used as models to understand how sensing and processing signals occur in other bacterial chemosensory pathways. However, the sequencing of numerous bacterial genomes and the advancing of cell biology techniques have revealed the existence of many bacterial Che systems largely divergent from the enteric model, in their genetic organization as well as in their function and localization in cells (10, 11, 35, 48, 85, 91, 94, 98, 104).

A chemosensory system is a modified two-component system decorated with proteins that have, altogether, the function of bringing the sensitivity of the system up to extreme limit levels (up to 5 nM for aspartate, in *E. coli*). The proteins common to two-component systems and the *E. coli* Che system are a histidine kinase, CheA, and a response regulator, CheY. In a two component system, the response regulator generally regulates gene expression through DNA binding, whereas its counterpart in the Che system, the CheY protein, has the function of directly communicating with flagellar proteins, such as FliM and FliN, in order to translate the initial perception of a signal into an adjustment of the cell movement (75) (Figure 2). Analogously to traditional two-component systems, CheY proteins receive phosphoryl groups from their cognate histine kinase, CheA in the Che system. CheA is not engaged in directly perceiving the chemical signal. This function is, in fact, relegated to four specialized chemoreceptors (Tar, Tsr, Trg, Tap), also named Methyl-accepting Chemotaxis Proteins (Mcps) for the presence of a methyl-accepting domain in their C-terminal cytoplasmic region (Figure 2) (61, 80). Signaling molecules, such as aspartate or maltose and serine, can directly bind the N-terminal region of a Tar or Tsr receptor homodimer, respectively. Otherwise, perception of the signal can also occur by indirect binding to periplasmic Ligand Binding Proteins of ATP-binding cassette (ABC) transporters. The latter is the case of the minor receptors Trg and Tap, binding sugars (D-ribose and D-galactose) and dipeptides (Pro-Leu), respectively (58). A fifth receptor, Aer, is an MCP-like protein involved in oxygen sensing.

An adaptor protein, CheW, favors the interaction between the Mcp and the CheA proteins. The binding to attractant molecules inhibits CheA autophosphorylation, thus reducing the phosphotransfer to the motor regulator CheY and, ultimately, allowing bacteria to maintain the same swimming direction towards attractant molecules through a counterclockwise rotation of the flagellar machinery. Conversely, when the receptor binds to a repellent molecule or when it encounters a decreased concentration of attractant, the receptor undergoes a conformational change that stimulates CheA autophosphorylation, phosphotranfer to CheY and, ultimately, a tumbling, which is the result of a brief switch of the flagellar rotation from counterclockwise to clockwise. The frequency of tumbling is a function of the change in the concentration of repellent or attractant molecules in the medium. Bacteria will reduce the tumbling frequency when they orient themselves in the "desired" direction.

In order to ensure rapid responses to minimal concentration changes of given compounds, the *E. coli* Che system utilizes adaptation, which derives principally from the activity of methylation enzymes, the accessory protein CheZ and motor sensitivity. Mcps are methylated and demethylated on four specific glutamate residues by methyltransferases (CheR) and methyesterases (CheB), respectively (Figure 2). CheB activity increases when the protein is phosphorylated by CheA. Mcp methylation and demethylation allow adaptation of the receptor to a persistent attractant stimulus. For example, in the presence of a persistent negative stimulus, the receptor induces the formation of CheA-P and in turn CheY-P, thus stimulating tumbling. CheA-P transfers phosphoryl groups to CheB. CheB-P mediates adaptation by demethylating active chemoreceptors and reducing their ability to activate CheA. This process decreases the concentration of CheY-P and reduces the tumbling frequency. Ultimately by reducing the CheA activity to pre-stimulus levels, the adaptation restores the pre-stimulus tumble bias and the system is ready to respond to an increase in the concentration of repellent.

Conversely in the presence of a positive stimulus such as an attractant, at the beginning CheA autophosphorylation is reduced and so are the concentration of CheY-P and the frequency of tumbling. However, if the positive stimulus persists, also CheB-P results reduced, thus favoring the presence of chemoreceptors in the methylated state. Methylation, though, increases CheA-P and resets it to the prestimulus state in order to decrease the sensitivity of the system and prepare it for an eventual further increase in the attractant concentration.

The signal transduction pathway is also provided of an accessory protein, CheZ, which increases the rate of CheY autodephosphorylation, allowing signal termination within sensing periods (Figure 2) (65). Additional adaptation mechanisms are achieved at the level of the

motor protein FliM. In fact, the number of FliM molecules increases in response to a decreased concentration of CheY-P, increasing motor sensitivity (100).

3.2. Polar Localization and more

The first milestone in the study of *E. coli* Mcp localization was published in 1993 by J. Maddock and L. Shapiro who, with the use of antibodies directed against Tar, established that this chemoreceptor formed clusters located at the cell poles (Figure 1B) (45). The presence of polar clusters of chemoreceptors was in agreement with the theory, formulated some years earlier by Berg and Purcell, that the small size of a bacterial cell makes spatial detection of gradients inefficient (7) and that *E. coli* cells adjust their swimming direction based on a "memory" that allows them to compare an actual concentration of a substance with the one sensed in the near past. This way of perceiving a gradient is termed *temporal sensing* and engages the adaptation mechanisms described above. Ultimately, bacteria cannot detect the absolute concentration of a compound but rather changes in its concentration.

Years later, fluorescence microscopy confirmed the observations made by immunogold labeling (Figure 1B) and also proved that the *E. coli* Mcp polar clusters contain not only Tar, but also the remaining four receptors (Tsr, Aer, Trg, Tap), CheA, CheY, CheZ. While the localization of CheA, CheY and CheZ is Mcp-dependent, the recruitment of Mcps at the cell poles is CheR, CheB, CheA, CheY and CheZ-independent, even if in the absence of these proteins the resulting clusters are less compact than wild type (32, 44, 45, 72, 76, 101).

Beside the major clusters at the poles, minor lateral clusters were also observed by immunoglod labeling and fluorescence microscopy (32, 45, 46, 73). Most recently, an ultrahigh-resolution light microscopy technique termed PALM (Photo-Activated Localization Microscopy), which provides images below the diffraction limit and allows the visualization of single molecules through cycles of photoactivation and photobleaching, was used to visualize fluorescently labeled Tar-mEos proteins in *E. coli* cells (21) (Figure 1B). Beside the polar clusters, small clusters formed by 10-100 receptors as well as single proteins were observed along *E. coli* cells (21). Data from Thiem *et al.*, suggest that lateral clusters are newly formed clusters that localize to specific periodic positions along the cell body, which mark future division sites (83).

Lateral clusters formation occurs in a two-step process. Initially, a stochastic self- assembly only due to free diffusion, protein concentration, distance from existing clusters and protein-protein interactions, without the involvement of cytoskeletal or anchoring factors, determines later cluster formation (21)(32)(84)(95). Then, clusters that encounter an association with hypothetical structures localized at future division sites, result immobilized, grow and become polar after several rounds of cell division (83)(84).

Cluster prepositioning at the future division sites is proposed to ensure that every newly divided cell has at least one cluster and can perform chemotaxis. An additional function of lateral clusters might be to enable effective chemotaxis in longer cell. In fact, at distances over 2 micrometers, the rate of signal transduction from the sensory clusters to flagellar motors becomes limited by diffusion of phosphorylated CheY (29, 69, 75, 90).

3.3. Cluster formation for a concert of signals

What is the benefit of Mcp clustering? One benefit could be to increase the efficiency of receptor activity by raising the local concentration of all pathway components (21). However, a more complex explanation must be used to explain Mcp cluster formation. Several lines of evidence indicate that cluster formation ensures 1) amplification of the signal and 2) collaboration between chemoreceptors for increased sensitivity. In an attempt to understand how chemotactic receptors perceive signals with both high sensitivity and a wide range of responses, in 1998, Bray *et al.* proposed a model in which receptors are organized in a highly ordered array and can switch randomly between high- and low-active conformations (Figure 1C). When a receptor in the array is bound to an attractant molecule, it will result in a low-activation state. This low-activity state of the receptor will be transmitted to a number of unoccupied receptors. Together, these receptors will result in a low-activation state, even if they are not bound to attractant molecules, and the initial signal will be amplified (13). Subsequent experimental studies confirmed this theory by showing that cooperative interactions between receptors exist both *in vitro* (37, 41) and *in vivo* (76, 77, 90).

Amazingly, roughly ten years after Bray *et al.* postulated their hypothesis on the existence of highly ordered arrays, these arrays were visualized by cryo-electron tomography, a technique that allows for subcellular structures to be preserved in their native state and observed in 3D with an approximate 5-nm resolution. In the past five years, cryo-electron tomography has been largely used to study the nanoscale organization of bacterial chemoreceptors (Figure 1B-C). Despite small differences at the level of the subcellular localization and cluster packing, chemotaxis clusters from different bacterial species including *E. coli* and *C. crescentus*, show a universal organization at the molecular level. By cryo-electron tomography, chemoreceptor arrays appear as two plates, parallel and adjacent to the cell membrane, with the one closer to the membrane being fainter than the distal one (14, 101). The fainter plate would be composed of Mcps, whereas the darker one of CheA and CheW (Figure 1B). A side view of the plates revealed an ordered hexagonal honeycomb-like arrangement containing 90-180 hexagons, which perfectly fit the previously described model for Mcp organization in trimers of dimers (15, 37). Each hexagon would represent a trimer of dimer unit, with a dimer of downstream regulators CheA and CheW positioned at the center of it (15, 33, 34, 101). Combinations of cryotomography and fluorescent microscopy (lFM) techniques confirm that the visualized structure is, indeed, the chemosensory array (14).

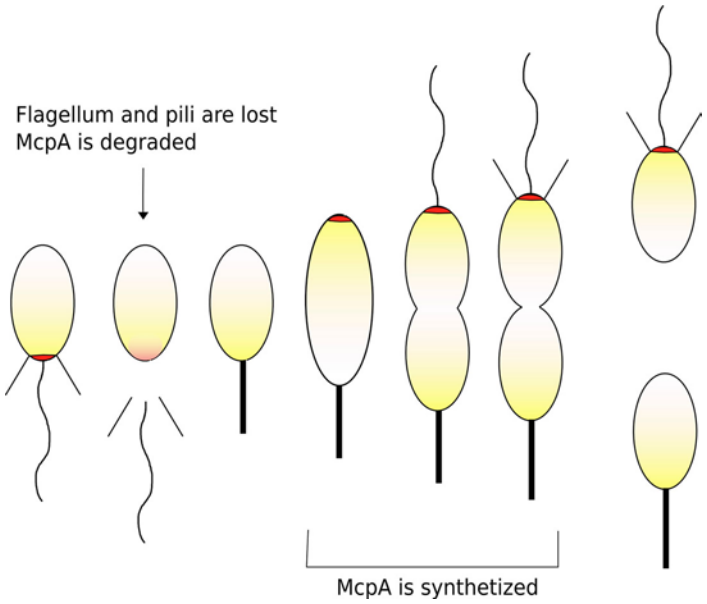

Figure 3. Schematic representation of *Caulobacter crescentus* cell cycle-dependent McpA (red) localization. At the beginning of the cell cycle, McpA is expressed and localizes at the flagellated pole. During the differentiation from swarmer to stalked cell, McpA is reduced and results absent in the stalked cell. When stalked cells start dividing, the McpA signal reappears at the opposite pole (future flagellated pole).

Detailed studies combining cryo-electron tomography, computational modeling and fluorescence resonance energy transfer (FRET) measurements, suggest a direct correlation between compactness of Mcp clusters and level of activity of these clusters in response to external conditions (33).

Beside signal amplification, MCP cluster formation also allows collaboration between receptors of different signal specificity, leading to a modulation of cell movement in response to a concert of integrated signals rather than a sum of each individual signal. As mentioned above, chemotactic clusters contain all five chemoreceptors. Chemoreceptors are not equally represented and in fact, Tar and Tsr are considered high abundance receptors, whereas Tap, Trg and Aer are present at roughly 10% level of Tsr and Tar (25). An example of collaboration between receptors consists in the so-called adaptational assistance neighborhood (42), in which high abundance receptors assist one another and low abundance receptors in achieving the methylation changes required to adapt to sensory stimuli (19, 26, 97). In fact, the adaptation enzyme CheR can only bind high abundance receptors containing the C-terminal NWETF pentapeptide (5, 6, 71, 99). However, the close proximity of high- and low-abundance receptors allows the low abundance receptors that do not have the pentapeptide, to be methylated by CheR bound to high-abundance receptors (Figure 2).

Collaboration between receptors also occurs at the level of signaling, since each receptor can not only transduce the signal coming from its own sensing periplasmic domain, but it can also transduce signals generated by neighboring receptors. The occurrence of collaborative signaling in clusters formed by different chemoreceptors was demonstrated by introducing single aminoacid substitution in Tsr, such that the receptor was still able to localize, form clusters and interact with other chemoreceptors, but was not capable of transducing a signal. *E. coli* cells only containing the mutated allele of Tsr could not respond to stimuli. However, the introduction of a wild type copy of Tar in the Tsr mutated strain rescued Tsr function, implying interactions and collaboration of Tsr and Tar in the highly ordered chemoreceptor team (3). Ultimately, the level of sensitivity to a stimulus is not determined by the abundance of the receptor engaged to perceive that stimulus, but by the number of interactions between receptors of that and other signal specificities (77).

4. CELL CYCLE-DEPENDENT LOCALIZATION

The first real study on Mcp localization was performed by Alley *et al.* on McpA, one of the 18 *C. crescentus* receptors, in immuno-gold labeling experiments (1). The study from Alley *et al.* revealed that gold particles generated by anti-McpA antibodies were located at the flagellated pole of swarmer cells (Figure 3).

C. crescentus cells differentiate during their life cycle into swarmer and stalked cells. The two forms are named after their ability to move or remain sessile, respectively. Swarmer cells contain polar flagella and pili that guarantee cell movement. Stalked cells contain an extrusion termed stalk important for nutrient intake during the sedentary life style (17). Movement occurs through the clockwise rotation of the single polar flagellum. The switch of rotation causes short reversals in the direction of swimming (36). Therefore, the biased frequency of

flagellar switches allows bacteria to orient themselves in a preferred direction. Che proteins regulate this phenomenon. *C. crescentus* possesses a single *che* operon and 18 Mcps.

The localization of the Che cluster at the flagellated cell pole might have two means: i) ensuring that swarmer cells, capable of performing cell movement, inherit a Che apparatus immediately after division (20) and ii) presumably, increasing the efficiency at which the Che apparatus modulates cell movement by minimizing the diffusion of CheY-P between Che system and flagellar motor and thus facilitating a rapid response.

CplXP-mediated degradation of McpA in the stalked cell eliminates McpA from this non-motile cell type (87). At the beginning of the cell cycle, McpA is expressed. Coincidently with the beginning of the differentiation from swarmer to stalked cell, McpA levels are reduced and the protein completely disappears when differentiation is completed (Figure 3). McpA degradation coincides with the loss of flagellum and pili. When stalked cells divide to generate a new swarmer cell, the McpA signal reappears (2, 87, 88) (Figure 3). Interestingly, it has also been shown that ClpXP-mediated proteolysis might be a general mechanism used by *C. crescentus* cells to segregate also other chemoreceptors during cell division. Indeed, the cytoplasmic chemoreceptor McpB, which localizes at the flagellated cell pole of the swarmer cell, also undergoes ClpX-mediated degradation in the stalked cell during the cell cycle. ClpX-mediated degradation might be mediated by a triaminoacid sequence (LAA) positioned immediately before the CheR docking site and common to all *Caulobacter* Mcps with a CheR docking site (McpA, McpB, McpD, McpH, McpR, McpI and McpP) (63, 87).

Recently, the enormous advancements of cell biology techniques allowed a better characterization of the Mcp architecture. Cryo-electron tomography showed that Caulobacter Mcps are localized in arrays that are not exactly at the pole but rather near the pole (100-300 nm from the flagellum) and always at the convex side of the cell (14, 34). This result suggests that i) the poles might be too crowded to accommodate the relatively large chemoreceptor arrays or that ii) the latter might recognize a convex curvature over a concave one.

5. BIMODAL LOCALIZATION: *Rhodobacter sphaeroides*

5.1. At the pole and mid-cell

Rhodobacter sphaeroides is a bacterium with a complex life cycle including aerobic, anaerobic and photosynthetic growth. It employs three chemosensory operons and a single flagellum to generate modulated cell movement towards attractants such as organic acids, oxygen and light. The three *che* operons contain a complete set of *che* genes, of which only two are expressed under laboratory conditions (61) and are essential for chemotaxis (62). Similarly, only one of two flagellar operons present in the genome is expressed in laboratory conditions to generate a single flagellum (60). Each *che* operon contains a gene encoding a Tlp (Transducer-Like Protein) cytoplasmic receptor (23, 62, 96). There are a total of four *tlp* genes on the chromosome. Thirteen genes for putative chemoreceptors are located elsewhere on the genome. Chemotaxis is generated by the biased frequency of stops of a single flagellar motor (61). During such stops, bacteria reorient (Figure 4A).

In 2002, through fluorescence microscopy analyses, Wadhams *et al.*, showed that the subcellular organization of the *R. sphaeroides* chemotaxis machineries displayed a much higher level of complexity compared to the enteric paradigm. In fact, *Rhodobacter* Che systems are organized in two major clusters, one forming at the cell pole and the second one localizing at the center of cells (92) (Figure 4A). While the polar cluster contains the McpG transmembrane receptor as well as the CheOp2 encoded CheA2, CheW2, CheW3, and CheR2, the cytoplasmic cluster contains the TlpC and TlpT cytoplasmic receptors and CheOp3 encoded proteins, CheA3, CheA4, CheW4, CheR3 (91–93). Localization of both polar and cytoplasmic receptors is affected by deletions of their respective CheA and CheW, more severely than in their enteric counterpart (32, 45, 72, 73, 94). Interestingly, neither of the CheAs is required for the formation of the cytoplasmic cluster (94).

The architecture of the polar clusters has been analyzed by cryo-electron tomography and shown to exhibit the typical array of trimers of dimers also observed in other bacterial species (15). However, how the cytoplasmic cluster is organized remains mysterious. Less mysterious is the mechanism by which *R. sphaeroides* cells ensure the segregation of the cytoplasmic cluster and the capability of each daughter cell to perform chemotaxis immediately upon division (85). Besides containing all components of the cytoplasmic cluster, *cheOp3* also contains a gene, *ppfA*, encoding a homolog of a bacterial type I DNA partitioning factor (ParA). Several pieces of evidence show that the PpfA protein is implicated in a plasmid segregation-like mechanism, ensuring the correct positioning and partitioning of the cytoplasmic clusters in dividing cells (66). In wild type cells, the single cytoplasmic cluster is stably localized at the center of cells; early during the cell cycle, it divides into two clusters each one localized at the second and third quadrant of cells, respectively (85) (Figure 4B). After cell division, these two clusters result partitioned between the two daughter cells and each one of them shows one cluster at mid cell (Figure 4B). In cells lacking PpfA, cells always show one cluster. After cell division, this cluster is inherited by one daughter cell; in the second cell, a cluster appears only 30-40 min after cell division. What Thomson *et al.* propose, is that in the absence of the PpfA partitioning factor, newly synthesized proteins all collect into one cluster and only after cell division and in the absence of an already existing cluster, they can form a new one. Therefore, it is not the amount of total protein that changes, but their correct partitioning into two clusters. This hypothesis is supported by the fact that, in elongated cephalexin treated cells, the *ppfA* cell single cluster is always brighter than any of the multiple periodic clusters present in wild type cells (85). Cells lacking PpfA are defective in chemotaxis (61). Most

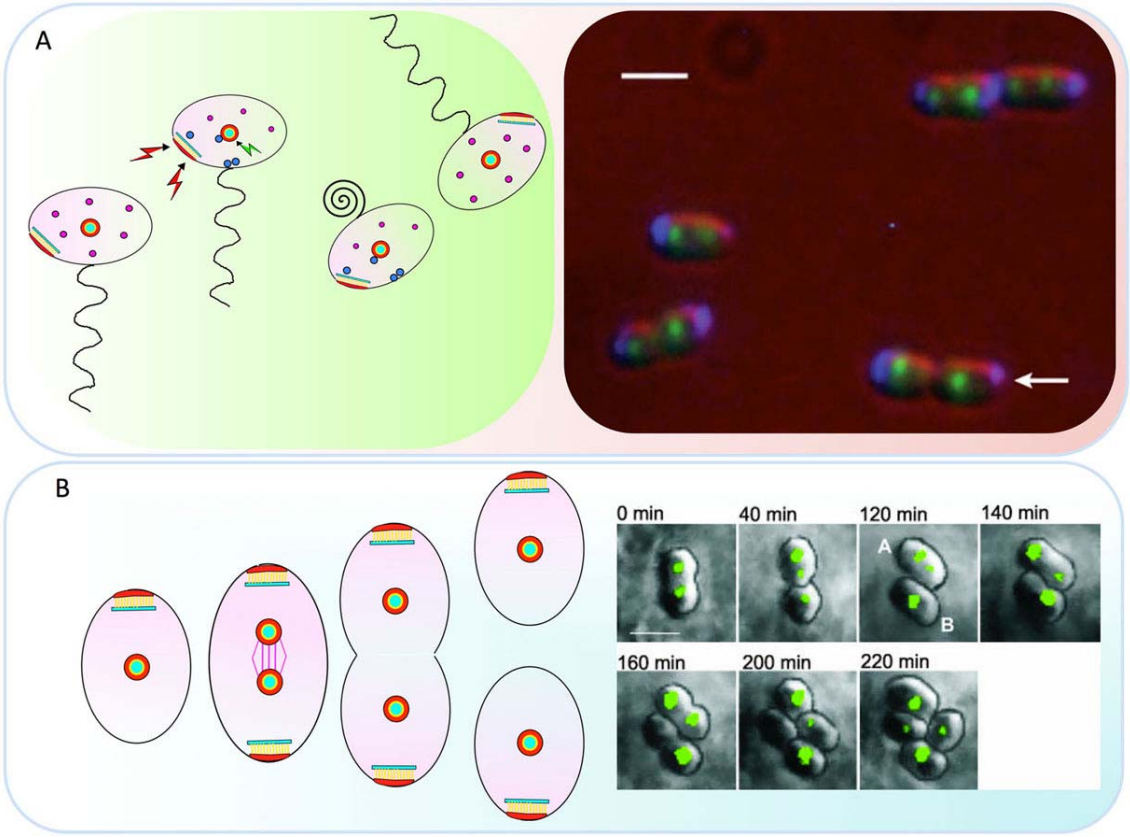

Figure 4. (A) (Left panel) Schematic representation of a *R. sphaeroides* cell swimming in a direction and responding to an increasing concentration of a chemical with a re-orientation and a consequent change of direction. Re-orientations are stimulated by a temporary accumulation of CheY-P molecules (blue; unphosphorylated CheY are represented in purple), which communicate to the single polar flagellar motor to temporarely stop the rotation. (Right panel) Localization, by fluorescence microscopy, of the polar chemotaxis cluster (CheW3-CFP, blue), the cytoplasmic cluster (CheW4-YFP, green). Membrane are in red. The arrows indicate dividing cells. This figure was reproduced from Porter *et al.*, Nature, 9(153-165), with permission of Nature Publishing Group. (B) (Left panel) Schematic representation of how the cytoplamic and the membrane clusters segregate in a *R. sphaeroides* dividing cell. Purple lines represent the ParA-like segregation factor, PpfA. (Right panel) Live fluorescence images of a cytoplasmic cluster (green) duplicating and segregating in a *R. sphaeroides* dividing cell. This figure was reproduced with permission of PNAS (85).

likely, this defect is due to the fact that a high percentage of cells within the population lack the cytoplasmic cluster upon division and is therefore incapable of performing chemotaxis (85). It would be interesting to prove this hypothesis by comparing the rate of chemotaxis in populations at different stages of growth.

The ATPase ParA works in partnership with the DNA binding protein ParB, in the binding and recognition of the centromere-like region *parS/parC* and in order to achieve plasmid segregation (62). Does PpfA, like ParA, have ParB-like partner proteins? It has been recently shown that the N-terminal domain of the TlpT cytoplasmic receptor, whose gene is encoded next to *ppfA*, has homology to ParB and that the partitioning of TlpT cytoplasmic clusters is coupled with chromosome segregation through PpfA nonspecific DNA-binding and interaction with the TlpT N-terminal ParB-like domain (66)(74). The fact that orphan *parA* genes have been identified in *che* operons of many bacterial species suggests that the segregation mechanism of the *Rhodobacter* TlpT cluster might be widespread in the prokaryotic world (66). A ParA-like segregation mechanism has also been shown to regulate the localization of polar Che clusters in *Vibrio cholera* (64).

5.2 How is a bimodal localization achieved and why?

In *R. sphaeroides* the presence of two clusters containing different receptors that are distantly positioned, one at the cell pole and one within the cytoplasm, most likely translates into the physiological need to segregate and transduce external and intracellular signals, respectively. Therefore, both clusters might be capable of temporally perceiving gradients formed by very distinct sets of signals. While attractants from the external environment are thought to be sensed by the transmembrane receptors, the cytoplasmic chemoreceptors might sense signals reflecting the metabolic state of the cell. The capability of sensing metabolic states is supported

by the fact that *R. sphaeroides* chemotactic responses require internalization of the attractants through transport.

Cells use different strategies, including high molecular specificity and transcriptional regulation mechanisms, in order to achieve the physical separation between polar and cytoplasmic clusters.

5.1.1. Protein specificity.

The CheA P5 specific domains, responsible for the coupling of the CheA to the CheW and the Mcp, determine the localization of CheA proteins at the pole or in the cytoplasm. Swapping experiments, in which the P5 domains of the cytoplasmic CheA3 and CheA4 were replaced with the one of the polar CheA2, caused the chimeric CheAs with the P5 domain from CheA2 to localize at the pole and the ones carrying the CheA3 or CheA4 P5 domain to localize in the cytoplasmic cluster (68). Chimeric CheAs can not complement the chemotactic defect of *cheA* deletion mutants, suggesting that individual pathways will only function when in the right subcellular region (68). Also, none of the *Rhodobacter* proteins can complement the deletion of a homolog localized to a different position in the cell. Deletions in components of one pathway resulted in Che proteins of that pathway becoming diffused in the cytoplasm, but never localized in the wrong cluster (94). The high specificity of these chemotaxis proteins contributes to the absence of cross talk between polar and cytoplasmic clusters (68, 74). An additional specificity checkpoint is represented by the specificity of phosphate flows from CheAs to CheYs established by the CheA P1 domains (68).

5.1.2. Transcription.

While transcription of *cheOp2* is regulated by the sigma factor 28, transcription of *cheOp3* is regulated by sigma 54 (47). The temporal separation in the transcription of the two sets of gene products might contribute to their physical separation. The separate regulation might also help in balancing the responses to extra- and intra-cellular signals under different growth conditions.

In *R. sphaeroides* chemotaxis response, it is thought that the cytoplasmic cluster senses the metabolic state of the cell and tunes the strength of the response to signals through the membrane cluster to produce a balanced response. This implies that, even if a physical separation between polar and cytoplasmic Che pathways might be essential to segregate the signals generated from the two pathways, an integration of these signals must also exist. The collaboration between the two signaling clusters could occur through a phosphorelay in which the cytoplasmic CheA3 phosphorylates CheB2, which in turn transfers the phosphoryl group to the polar CheA2. CheA2 can then phosphorylate any of its cognate response regulators (86). This way, all six response regulators receive signals directly from the polar cluster or indirectly from the cytoplasmic cluster. Similarly, CheA2 phosphorylates CheB2, which in turn demethylates Tlp receptors (74). Ultimately, cell behaviors should result from a concert of integrated signals.

6. ARCHITECTURE OF CHEMOSENSORY MODULES IN A NON-FLAGELLATED BACTERIUM: *Myxococcus xanthus*

6.1. Frz system and directional control

As described above, chemotaxis has been mostly studied in flagellated bacteria, where the biased alternation of smooth swimming and tumbling or smooth swimming and pausing, modulates cell movement. The gliding bacterium *M. xanthus* lacks flagella and produces, instead, two distinct motility machineries that utilize Type IV pili (S motility) or distributed surface adhesion complexes (A motility) (49). Which of the two motility systems is turned on depends on cell density and features of the surface where bacteria are moving. Motility is essential to accomplish complex social behaviors such as predation and fruiting body formation (9, 30, 31, 70). In addition to the two motility systems, genome searches revealed the presence of eight operons encoding chemosensory proteins and 13 additional *mcp* genes scattered in the chromosome (49, 104), suggesting a regulation of cell movement in response to the environment. If so, how would regulation of cell movement occur when motility is produced through non-flagellar motility apparatus?

While gliding on solid surfaces, *M. xanthus* cells reverse the direction of their movement with a certain frequency. Cells modulate the reversal frequency in response to external conditions. Control of cell reversals and coordination of the two motility systems is operated by a set of chemotaxis proteins encoded by the *frz* operon. It is hypothesized that the ability to reverse direction allows cells to periodically reorient themselves similarly to how, changing the rotation of flagella in enteric bacteria, causes tumbles and cellular reorientation. However, a biased movement of *M. xanthus* individual cells towards known "chemoattractants" has never been observed. On the other hand, results suggest that it is more likely that the Frz system mediates a tactic response that occurs at the group level rather than at the level of individuals (9, 18, 81). For example, it has been shown that *M. xanthus* cells that encounter a prey colony start moving in a unique fashion characterized by the formation of waves made of thousands of cells that move forwards through the prey colony and periodically reverse in a perfectly synchronized manner. These waves of cells are called ripples and are hypothesized to be required to achieve maximum lysis of the prey colony. Ripple formation requires Frz functions. Ripple frequency and "wave length" change with time with a trend that resembles adaptation patterns. Indeed, frequency and "wave length" directly depend on the FrzG and FrzF (CheB and CheR, respectively) activities. Because of similarities to chemotaxis, this behavior was termed *predataxis* (9). It has been also shown that while *M. xanthus* individual cells only exhibit non-vectorial displacements, large groups of cells are capable of moving towards a two-dimensional gradient, suggesting that chemotaxis requires cell contacts and coordinated motility (81). How cells communicate within the group to synchronize cell movement and respond to external signals as a group rather than as individuals might involve the

cytoplasmic chemoreceptor of the Frz pathway (see below) (48).

frz genes were firstly discovered in a screen for mutants defective in aggregation during development (103). The *frz* denomination was inspired by the fact that cells failed to form discrete mounds and rather aggregated in "frizzy" filaments that would never develop into fruiting bodies (103). The developmental defect of *frz* mutants is due to the inability of cells to control their reversal frequency during gliding motility (12). Wild type cells reverse their direction of gliding about every 7 minutes when plated at very low density on starvation media. In contrast, most *frz* mutants reverse the direction of their movement every hour (12), whereas some constitutively signaling *frzCD*^{c} reverse more often than wild type. Although *frz* mutants are capable of performing both A and S motility (12), the unregulated reversal frequency does not allow cells to coordinate their movement when they are in large groups. Therefore, vegetative swarming of *frz* colonies is defective and reduced as compared to wild type (12).

The *frz* operon includes a gene encoding a cytoplamic Mcp, FrzCD (4, 51–53, 67); two genes encoding CheW proteins, FrzA and FrzB (16); a fusion between a CheA and a CheY domain, FrzE (16, 27, 54); a CheR and a CheB, FrzF and FrzG, respectively (16, 67). Additionally, a divergently transcribed gene encodes a protein with two CheY domains, FrzZ (16, 28).

FrzCD, FrzA and FrzE constitute the core of the Frz pathway, as they are all important for vegetative swarming, development and response to some known repellents such as iso-amyl-alcohol (16). FrzB, FrzF, FrzG and FrzZ are required for vegetative swarming and development (16). FrzCD is an unusual chemoreceptor as it lacks the transmembrane and periplasmic domains typical of enteric Mcps. While the unique N-terminal region of FrzCD has been shown to be important in the regulation of A motility (see below) and its deletion only results in minor defects in S motility and development (16, 50), the methylation of the conserved C-terminal domain has been shown to play a central role in regulating cell reversal frequency, as well as S motility and development (4, 16, 67).

The Frz pathway regulates the reversal frequency of the A- and S-motility machineries by modulating the frequency of pole-to-pole oscillations of motility proteins (38, 55, 56). Two recent studies show how the Frz system establishes such dynamic polarity in cells through its activity on the GTP/GDP cycle generated by MglA/MglB (39, 102). MglA is a small GTPase of the Rho family, which accumulates in its GTP-bound state at the leading cell pole (39, 102). MglA determines the polar localization of A- and S- motility proteins, essential for the functioning of both motility systems. MglB, a GAP (GTPase Activating Protein)-like, localizes at the rear of cells inhibiting MglA positioning at this site. By releasing MglB from the back pole and allowing MglA-GTP accumulation at this site, the Frz system acts as a GEF (Guanine nucleotide Exchange Factor) and promotes a reversal (39, 102).

6.2. FrzCD localization and cell-cell communication

When Mauriello and coworkers first localized FrzCD, fluorescence microscopy analyses revealed a localization pattern very different from the ones previously observed for transmembrane and cytoplasmic receptors. In fact, the combination of cytological analyses performed *in vitro* by using anti-FrzCD antibodies and *in vivo* with the use FrzCD-GFP fusions, showed that FrzCD forms cytoplasmic clusters that appear helically arranged and span the cell length (48). FrzCD clusters are dynamic and their number, size and position vary in cells. FrzCD-GFP never localizes at the poles. Interestingly, the number of FrzCD clusters was correlated with cellular reversal frequency: fewer clusters were observed in hypo-reversing mutants and additional clusters were observed in hyper-reversing mutants. Also clusters are more diffused in mutants lacking the CheA (FrzE) function. One unexpected and exciting finding is that *M. xanthus* cells making side-to-side contacts show transient FrzCD cluster alignments (48) (Figure 5A). Detailed statistical analyses show that alignment occurs at the moment of side-to-side contacts and is maximal when cells are fully aligned. Cluster alignment is not observed in strains lacking the histidine kinase FrzE, indicating the requirement of feedback regulation mechanisms from the Frz pathway (48).

What is the function of the FrzCD cluster alignment? Interestingly, in the same study, side-to-side cell contacts have been shown to influence not only the organization of the clusters but also the timing between cell reversals. In fact, converging cells making side-to-side contacts exhibit increased cellular reversals (48) (Figure 5B). These reversals are not seen in *frzCD* mutants (16). Because i) side-to-side cell contacts influence FrzCD localization, ii) FrzCD controls cellular reversal frequency and iii) side-to-side contacts stimulate cell reversals, the authors suggested a model in which FrzCD clusters are part of sensing structures devoted to perceive the presence of neighboring cells and modulate the reversal frequency in response. Interestingly, the structure which senses the presence of neighboring cells might identify with the A-motility focal adhesion complexes (43, 56, 79). In fact, it has been shown that in an *aglZ-yfp* and *frzCD-gfp* expressing strain, AglZ-YFP and FrzCD-GFP clusters clearly localize in an exclusive manner. In particular, AlgZ occupies positions that are never occupied by FrzCD and *vice versa* (50) (Figure 5B). Therefore, it is not to exclude the hypothesis that A-motility adhesion-like complexes recognize each other in adjacent cells through their outer membrane portions (43). A recruitment of AglZ-YFP occurs at the sites of recognition, which are aligned in adjacent cells, and exclusion of FrzCD-GFP from these sites caused them to localize at positions also aligned (Figure 5B). Such involvement of AlgZ in FrzCD cluster alignment could be tested by analyzing the FrzCD cluster alignment in cells lacking AglZ and also by checking whether AglZ cluster also align in adjacent cells.

Contact-dependent cellular reversals may be important for coordinated cell movements, such as

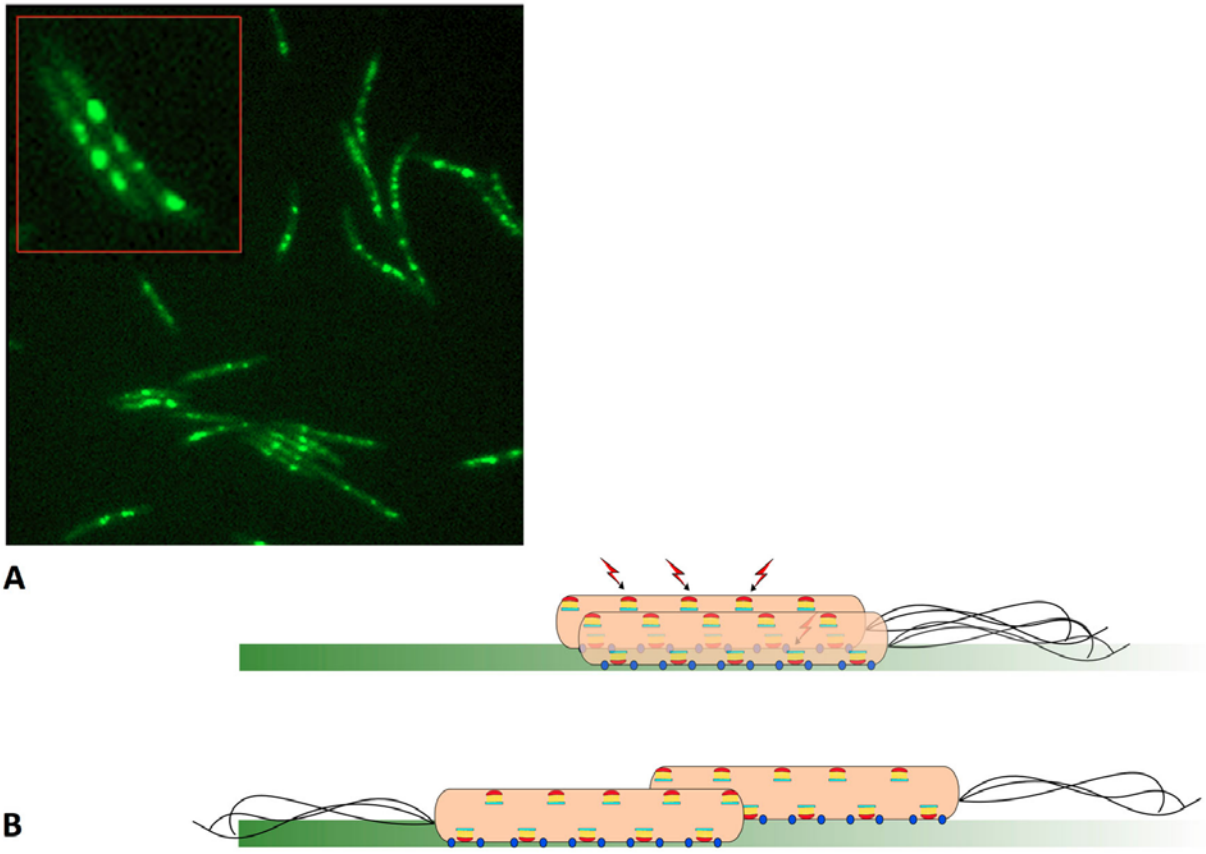

Figure 5. (A) FrzCD-GFP clusters (green) align in adjacent cells. (B) Schematic representation of FrzCD-GFP clusters stimulated by cell contacts and aligning. The stimulus generating from the cell contact is translated in the two adjacent cells and one or two of them will reverse the direction of movement in response. FrzCD-GFP cluster alignment might occur inderectly and be driven by the alignment of focal adhesion complexes in adjacent cells.

"rippling", the wave-like periodic movements associated with predation and fruiting body formation (9, 70).

6.3 Multiple clusters for spatial sensing

Results suggest that *M. xanthus* FrzCD clusters are directly involved in a cell contact dependent-communication mechanism, as they respond, by rearranging their intracellular distribution, to full contacts between adjacent cells, a phenomenon that might also favor coordinated cell behaviors (48). FrzCD is also involved in the regulation of predataxis, a predator prey contact-dependent chemotaxis-like behavior (9). The ability to respond to the presence of adjacent siblings or prey via a chemosensory apparatus and in a contact-dependent manner indicates that *M. xanthus* is capable of perceiving a change in a given signal at different positions along the cell body, namely a spatial sensing. Certain filamentous cyanobacteria exhibit phototaxis by spatially sensing the light gradient across the cell (22). However, spatial sensing of chemical gradients has never been observed in prokaryotes.

The vibrioid microaerophilic bacterium *Candidatus Thioturbo danicus* (57) possesses two sets of flagella, each at a different cell pole. Flagellum rotation allows these bacteria to swim towards the optimal concentration of oxygen. Experimental data strongly suggest that the type of swimming engaged by this bacterium, characterized by translation along and rotation around their short axis, cannot be explained as driven only by temporal sensing. The oxygen concentration in the environment must be sensed at either cell pole and, as result, the bundle of flagella at the end of the cell exposed at the higher oxygen concentration will rotate at higher speed than the flagella exposed at the lower concentration. This difference in speed would enable the bacterium to bend its swimming path away from high oxygen concentration (82).

In *E. coli*, multiple minor clusters have been detected with traditional as well as with more sophisticated techniques (21, 45, 73). Despite the presence of multiple minor clusters, it is unlikely that *E. coli* cells can detect the environment in a spatial manner, as its longer axis measures less than 2 μm and integration of the signals between these clusters surely occurs. Multiple clusters might have evolved in other bacterial species, such as *M. xanthus*, to generated a spatio-temporal response. Also, such response would be more appropriate in *M. xanthus* natural environments where macromolecules, prey and

more in general compounds generating steep gradients represent the most abundant source of nutrients.

7. SUMMARY AND PERSPECTIVES

How does the number and positioning of Mcp clusters translate into bacterial cell behaviors? *E. coli* bacterial cells are too small to be able to sense a difference in the concentration of a chemical along their body (7). Therefore, two clusters are sufficient to respond to temporal chemical gradients with maximum efficiency and, through the diffusible CheY, to modulate the activity of the multiple peritrichous flagella.

In *R. sphaeroides* the presence of two clusters containing different receptors distantly positioned, one at the cell pole and one within the cytoplasm, most likely translates into the physiological need to segregate the perception of external and intracellular signals, respectively. Therefore, both clusters might be capable of temporally perceiving gradients formed by very distinct sets of signals. Together with the fact that the two clusters are differently localized, the high specificity of the chemotaxis proteins in them contained also contribute to ensure the absence of cross-talking between the two. While the *R. sphaeroides* polar cluster might segregate like *E. coli* upon cell division, the cytoplasmic cluster possesses its own specific segregation machinery to ensure that each daughter cell can perform chemotaxis immediately after division. Such machinery has been shown to be associated with Che systems of many other bacteria suggesting that is widespread in nature (24).

M. xanthus cells also have multiple MCP clusters. Unlike *R. spheroides,* such clusters show the same receptor composition and, therefore, are expected to respond to the same signals and behave in the same way within cells. What is the reason, then, of having multiple clusters? Considering that numerous behaviors requiring the Frz chemosensory system, are mediate by cell contact in *M. xanthus*, and considering that *M. xanthus* responds to steep changes in concentrations rather than gradients of macromolecules and preys, we hypothesize that i) *M. xanthus* might be capable of spatial sensing and ii) it might regulate cell movement with very different mechanisms than chemotaxis.

Ultimately, a link must exist between the abundance and architecture of chemoreceptors and the number of functions and behaviors that a bacterium can perform.

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