

CELLULAR TARGETTING AND SEGREGATION OF BACTERIAL CHEMOSENSORY SYSTEMS

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ABSTRACT

The bacterial cytoplasm is not a homogeneous solution of macromolecules, but rather a highly organized and compartmentalized space where the clustering and segregation of macromolecular complexes in certain cell regions confers functional efficiency. Bacterial chemoreceptors represent a versatile model system to study the subcellular localization of macromolecules, as they are present in almost all motile bacterial and archaeal species, where they tend to form highly ordered arrays that occupy distinct positions in cells. The positioning of chemoreceptors clusters, as well as their segregation mechanism on cell division, varies from species to species and probably depends on cells size, environment and speed of movement. In this review we summarize the current understanding of the architecture and the segregation mechanisms of chemoreceptors in a limited number of bacterial model systems, mainly *Escherichia coli*, *Bacillus subtilis*, *Rhodobacter sphaeroides* and *Myxococcus xanthus* and suggest that the pattern of chemoreceptor distribution is coupled to behavioral life-style of that species.

INTRODUCTION

The ability of all organisms to perceive changes in the environment and adapt their behavior in response is essential feature for survival. Bacteria have evolved several systems to sense external stimuli and modulate cellular responses to adapt their metabolism and physiology to new environments. Two-component signaling systems represent the best-known bacterial molecular system for detecting and responding to signals. In such systems, the perception of signals usually results in the modulation of gene expression through the autophosphorylation of a membrane bound histidine kinase, which in turns transfers the phosphoryl group to the aspartate of a soluble response regulator. Two-component systems generally respond to a single environmental signal and switch between active and inactive states. Chemotaxis or chemosensory systems (CSS) are modified two-component systems that during evolution acquired accessory modules, conferring new functions that increased the range of responses and sensitivity of the system. CSS can perceive multiple signals thanks to the presence of multiple specialized receptors. The organization of these receptors appears to be common across species and allows the amplification of the initial signal. Specific enzymes in the pathway periodically reset the system to a *prestimulus* state, a property termed adaptation, which allows a rapid response to changing and new signal concentrations. Beside motility, CSS can modulate a large variety of functions, such as cyst formation (Berleman and Bauer 2005), biofilm formation (Corral-Lugo *et al.* 2015), and, occasionally, gene expression like canonical two-component systems (Kirby and Zusman 2003).

A common feature of chemosensory proteins is their ability to form highly ordered structures which look like intricate honeycomb-like lattices when examined using cryo-electron microscopy (Studdert and Parkinson 2005; Li *et al.* 2011; Briegel *et al.* 2012a; Liu *et al.* 2012). While this hexagonal array appears universal among motile Bacteria and Archaea (Briegel *et al.* 2009, 2015), the subcellular localization and distribution of CSS can vary between different bacterial species, probably reflecting their different lifestyles and behaviors and the different functions of the CSS. The fusion of fluorescent proteins to chemoreceptors has allowed them to be resolved as discrete fluorescent clusters located in different cell regions, depending on the bacterial species, such as the cell poles or the nucleoid (Maddock and Shapiro 1993) (Moine *et al.*, 2016). How CSS are targeted to their final cell location and, when the proteins are localized to a discrete site, how are they segregated between daughter cells? In this review, we will review some recent finding on the cellular positioning of chemoreceptors in

well-characterized bacterial model systems, such as *Escherichia coli*, *Bacillus subtilis*, *Rhodobacter sphaeroides* and *Myxococcus xanthus*. We will then discuss how these localization mechanisms might allow the correct segregation of these macromolecular complexes on cell division.

BACTERIAL CHEMOSENSORY SYSTEMS

The *E. coli* and *B. subtilis* Che system: composition and signal transduction

E. coli, like many bacteria, moves through its environment using the rotation of helical flagella (typically 4-6). When swimming, the flagella all rotate in a counter-clockwise (CCW) direction coming together in a bundle (Figure 1A). The rotation is generated by transmembrane motors at the base of the flagella powered by either the proton or sodium motive force, depending on the species. Cells move towards favorable conditions using a biased random walk, changing direction more often when conditions are worsening and less often when they are improving (Berg and Brown 1972) (Figure 1A). These reorientations come about when one or more of the flagellar motors change the direction of rotation to clockwise forcing the flagellar bundle apart and the cell to tumble on the spot, a behavior also termed *tumbling* (Darnton *et al.* 2007) (Figure 1A). When all flagella resume CCW rotation, the bundle reforms and the cell swims smoothly in a new direction.

This switch in direction of flagellar rotation in response to changing environmental conditions is controlled by the chemotaxis pathway (Figure 1B). Transmembrane receptors, known as methyl-accepting chemotaxis proteins (MCPs), detect the presence of attractants and, probably, repellents via sensing domains in the periplasm. Binding of these effectors results in a conformational change, which is transmitted through the inner membrane to the cytoplasmic tip of the receptor (Falke and Erbse 2009). The conformational state of the receptor controls the activity of a histidine kinase, CheA, which binds to the cytoplasmic tip of the receptors, along with an adaptor protein CheW. When conditions are improving, i.e. increasing attractants or decreasing repellents, the receptors are in a state which inhibits the kinase activity of CheA. The converse is true when conditions are worsening, resulting in high CheA activity. CheA can phosphorylate two response regulators, CheY and CheB. When phosphorylated, the affinity of CheY for CheA decreases and it is released and diffuses in the cytoplasm. The affinity of CheY-P for the FliM protein of the flagellar motor increases and on binding it induces a change in rotation from CCW to CW resulting in a tumbling event (Figure 1B) (Wadhams and Armitage 2004). CheZ rapidly dephosphorylates CheY-P, terminating the signal and preventing the cell from tumbling for a prolonged amount of time (Figure 1B).

To reset the signaling state of the receptors, specific glutamate residues on the MCPs are demethylated by the phosphorylated form of CheB. This decreases the sensitivity of the system by decreasing the MCPs ability to activate CheA. CheB's action is opposed by a

methyltransferase, CheR, which constitutively methylates the glutamates (Hazelbauer, Falke and Parkinson 2008). Thus, the longer the system goes without being activated the more sensitive it becomes while after activation it is desensitized. This adaptation system allows cells to respond to a large range in concentration changes. Hundreds to thousands of copies of each protein ensures a flexible sensory system, able to respond to small percentage changes in effector concentration over a wide range of background concentration.

While *E. coli* represents the paradigm for the study of Che systems in Gram-negative bacteria, *B. subtilis* is the paradigm for Gram-positive bacteria. Like *E. coli*, *B. subtilis* also swims using peritrichous flagella, with flagellar activity modulated by a Che system as in *E. coli*, however the phosphorylation reactions give the opposite response. Increasing attractants or decreasing repellents induce the accumulation of phosphorylated CheY. Moreover, the CheY-P binding to the flagellar motor reduces, instead of increases, tumbling (Garritty and Ordal 1995) (Figure 1C).

While CheZ is absent in the *B. subtilis* Che system, indeed in all non-enteric species, two additional adaptation modules are present: CheC-CheD and CheV (Figure 1C). CheD has two activities. It deaminates the chemoreceptor methylation domain producing a positive charge that favors CheR/CheB binding to this region and therefore enhancing the adaptation reactions. It also forms a ternary complex with CheC and CheY-P where it induces the dephosphorylation of CheY-P by CheC, thus increasing tumbling. CheV is a fusion between a CheW and a response regulator domain. It has been proposed that the phosphorylation of this response regulator domain causes a conformational change in the CheW domain that inactivates CheA (Rao, Glekas and Ordal 2008).

***Rhodobacter sphaeroides*: keeping the outside in tune with the inside**

Unlike *E. coli*, which has a single copy of each of the chemotaxis gene (excluding the MCPs) the *Rhodobacter sphaeroides* genome contains genes for multiple copies of each component of the signaling pathway (Hamblin *et al.* 1997). Bioinformatics analysis has shown that many other bacterial species also contain multiple chemotaxis homologues (Hamer *et al.* 2010; Collins, Lacal and Ottemann 2014).

In *R. sphaeroides* the majority of these chemotaxis genes are organized in three operons, only two of which are expressed under normal laboratory conditions. The products of the two

expressed operons form spatially distinct signaling pathways, one localizes with the transmembrane receptors, very much like *E. coli*, while the other localizes to the cytoplasm with soluble chemoreceptors (Figure 2A). *In vitro* phosphotransfer experiments showed the potential for crosstalk between the two clusters exists, with the CheA localized with the transmembrane MCPs able to phosphorylate all the chemotaxis response regulators while the split kinase, CheA3/CheA4, of the cytoplasmic cluster can only phosphorylate CheY6 and CheB2, encoded in the same operon (Porter and Armitage 2002). However the extent to which the crosstalk actually occurs *in vivo* and its importance are unclear. Six *cheY* genes are present in the genome, but only two are needed for functional chemotaxis, CheY6 and either CheY3 or CheY4 (Porter *et al.* 2006) (Figure 2A). *R. sphaeroides* does not have a homologue of the *E. coli* phosphatase CheZ, however *in vitro* work has shown that CheA3 can both phosphorylate and dephosphorylate CheY6. CheA3 lacking the phosphatase domain cannot support chemotaxis *in vivo* (Porter *et al.* 2008). This suggests that the phosphatase activity may be required for signal termination. The requirement for either CheY3 or CheY4 is unclear, but probably reflects competition between signals from the external environment needing to compete with metabolic signals for a balanced response. Signal termination of CheY3 and CheY4 may use a phosphate sink, as identified in another alpha proteobacterium, *Sinorhizobium meliloti*. *R. sphaeroides* has a single, randomly positioned, flagellum which rotates CCW to push the cell and, rather than switching rotational direction stops periodically. Brownian motion reorients the cells during stops, ensuring the cell is swimming in a new direction when it starts rotating again. The need for two pathways both feeding into a single motor is still unclear, but it has been suggested that it may be to integrate the internal metabolic state of the cell with the external conditions when controlling cellular movement. (Figure 2B)

***Myxococcus xanthus*: modular organization of multiple Che systems**

Even more extreme is *M. xanthus* whose genome contains even more genes encoding Che proteins even though it does not swim. Instead, it moves on solid surfaces with the aid of two genetically distinct motility systems: a Type IV pilus-mediated (TFP) motility which also requires the production of exopolysaccharide (EPS); and a second motility system that uses internal focal adhesion complexes (FAC) to power motion (Figure 3A). In addition to eight operons containing complete sets of *che* genes, *M. xanthus* also encodes 21 MCP and several

orphan Che proteins (Figure 3B). Frz (Che1) and Che7 are cytoplasmic chemosensory systems. Data suggest that Che4, Che5 and Che6 interact to form a large chemosensory module regulating fruiting body formation (Moine et al., 2014). Che3, Che7 and Che8 also have functions during development, but while the actual roles remain unknown for Che4-Che8, Che3 is known to regulate the expression of genes important for fruiting body formation. The Dif (Che2) system is involved in the activation of EPS production, thus having an essential function in the TFP-mediated motility. Lastly, the Frz (Che1) system modulates the frequency with which cells periodically reverse the direction of their movement on solid surfaces to reorient, similar to controlled tumbles in *E. coli* (Figure 3A). Because of its function in the regulation of cell directionality, the Frz system is considered the analogue of the *E. coli* Che system.

THE Che PROTEINS ARE ORGANIZED IN HIGHLY ORDERED STRUCTURES

The unique properties of chemosensory systems result from their universal macromolecular architecture. Using high-resolution cryoelectron microscopy, Che proteins appear organized in an intricate 2D honey comb-like structure in which hexagonal shapes are regularly placed with a 12 nm packing distance (Zhang *et al.* 2007; Briegel *et al.* 2009). The hexagons are made of six MCP trimers-of-dimers networked by CheA-P5 domain/CheW rings (Figure 4A), the minimal functional unit consisting in two receptors trimers-of-dimers, a CheA dimer and two CheW (Figure 4A and B). Trimers can contain different receptor homodimers that associate via interactions at conserved residues in the cytoplasmic domain. It has been shown that in addition to CheA-P5/CheW rings, the receptor hexagons can be connected by rings composed of only CheW (Piñas *et al.* 2016) (Figure 4A), but whether these have a biological function is yet to be determined.

This architecture is not strictly required for signal transduction, as one functional unit is enough to generate phosphorylated CheY (Francis et al., 2002; Li et al., 2011; Li and Hazelbauer, 2011; Piñas et al., 2016). However, MCP clustering is essential to ensure the amplification of the initial signal and cooperation between chemoreceptors to reach the maximum sensitivity (Sourjik and Berg 2004; Ames and Parkinson 2006; Li and Hazelbauer 2014; Piñas *et al.* 2016). The packing of multiple receptors into a single array also aids the balancing and integration of signals from multiple signals.

The hexagonal organization is common to both transmembrane and cytoplasmic receptors from Archaea and Bacteria, with some small differences (Briegel *et al.* 2015). The transverse view of transmembrane Che lattices show two plates, parallel and adjacent to the cell membrane, the plate closer to the membrane being composed of MCPs, whereas the distal baseplate of CheA and CheW (Figure 4C). Cytoplasmic MCPs from *R. sphaeroides* and *V. cholerae* however are arranged in two hexagonal arrays of trimers-of-receptor-dimers sandwiched between two CheA and CheW baseplates (Figure 4D). Thus, receptors lacking a membrane-binding domain can self-associate through their N-terminal sensing domain (Briegel *et al.* 2014, 2016).

While the described architecture is highly conserved, the cellular localization of chemoreceptors as well as the determinants of their targeting varies in different bacterial species. The labeling of MCP and Che proteins using fluorescent fusions allowed the chemosensory systems to be resolved as discrete clusters and their positions and dynamics tracked.

CELLULAR LOCALIZATION AND SEGREGATION OF CHEMOSENSORY ARRAYS

Localization in *B. subtilis* and *E. coli*

The polar localization of chemoreceptors was first described over 30 years ago (Alley, Maddock and Shapiro 1992; Maddock and Shapiro 1993) and it is common to many bacterial species. Nevertheless, results from different research groups show that the polar targeting of chemoreceptors can be driven by very different mechanisms and molecular determinants.

A recent work by Hamoen and colleagues show that the polar localization pattern of the TlpA transmembrane chemoreceptor from *B. subtilis* is a consequence of its recruitment to the cell division site, which will later become the cell pole of the daughter cells (Strahl *et al.* 2015) (Figure 5A and B). TlpA targeting to the division site is driven by curvature recognition. In most *B. subtilis*-like Gram-positive bacteria, cell division involves the formation of a cross-wall, which generates membrane curvature at mid cell. Such curvature is much stronger than that of the cell poles, which does not differ significantly from the lateral curvature. Fluorescence microscopy shows that TlpA is recruited at the cell division site when the membrane starts to invaginate. Even after division is complete, the fluorescent signal remains on both sides of the division plane, at the level of the maximum curvature, and is not found at the central region of the septum where curvature is absent. Interestingly, the accumulation of TlpA at strongly curved regions is a direct consequence of the physical conformation of the receptor trimers of dimers. The individual dimers in the trimers are not parallel but form a *tripod-like* structure with a precise curvature, whose radius from the membrane plane to the cytoplasmic tip is very similar to that measured for the base of the cell division septum (37 nm vs 47 nm) (Figure 5C). The division site therefore represents an energetically favorable environment for the TlpA trimers of dimers because of the reduced membrane stress. Point mutations in TlpA key residues responsible of the formation of receptor trimers prevent the TlpA accumulation at division plane. Similarly, the insertion of a glycine stretch at the interface between the receptor transmembrane region and the cytoplasmic domain generates flexibility in the dimers such that that the cytoplasmic tip of the array can adopt a curved arrangement despite a lack of curvature at the transmembrane level (Figure 5C). These trimers became curvature-insensitive and localized anywhere in the cell membrane.

While in *B. subtilis* chemoreceptors exclusively localize at the cell poles and at the division sites, the *E. coli* MCPs accumulate at the cell poles, but they also nucleate in small lateral clusters, suggesting that in this bacterium curvature recognition might not be responsible or, at least is not the only determinant of receptor localization. The small lateral clusters are visible most in naturally or artificially elongated cells and when chemoreceptors are overexpressed (Thiem and Sourjik 2008) (Figure 6A). The number of clusters increases linearly with the cell length with an approximate 1 μm distance between clusters, suggesting that there is a minimal distance at which new clusters can form (Figure 6B). Interestingly, by controlling the receptor expression levels in cells, Thiem and colleagues showed that the cluster density (the number of clusters normalized to the cell length) largely varies in individual cells for each given receptor protein level (Thiem and Sourjik 2008). This result suggests that the nucleation of *E. coli* chemoreceptor clusters might occur stochastically in the membrane rather than at specific binding sites. The stochastic self-assembly model proposed by Thiem and Sourjik implies that receptors can spontaneously nucleate new clusters, but if an existing cluster is positioned at a critical distance, receptors are more likely to collide with and fuse with this existing cluster (Thiem and Sourjik 2008). Photoactivated localization microscopy also showed that the distribution of cluster sizes is continuous suggesting that large clusters are formed by the gradual absorption of smaller clusters and single proteins (Greenfield *et al.* 2009). The critical distance between receptor clusters might depend on the diffusion “freedom” and, thus, on the receptor level in cells, being smaller at high receptor concentrations and larger at lower concentrations. The existence of a limiting critical distance in cluster formation is also supported by the fact that, at very high receptor levels, the number of clusters reaches the saturation while their fluorescence intensity increases (Thiem and Sourjik 2008).

The stochastic self-assembly model does not exclude that, beside the spontaneous formation of receptor clusters, chemoreceptors are also recruited at some specific sites in the membrane where they might nucleate new clusters. Lateral clusters might become polar after one or more rounds of division, when the cluster localization site becomes the cell division site and later a cell pole (Figure 6B). Polar localization might be further stabilized by the effect of the curved membrane (Strahl *et al.* 2015) or by a preference for the lipid composition of the poles (Mileykovskaya and Dowhan 2000). This systematic accumulation at the cell pole would explain the fact that the receptor fluorescence intensity at the cell pole is always higher than

that of lateral clusters. Possibly, lateral clusters might ensure that in *E. coli* long cells the regulation of distant flagellar motors is not limited by the CheY-P diffusion.

Strahl and colleagues propose that the different mechanisms seen in *E. coli* and *B. subtilis* receptor localization might be explained by the different curvatures, as *B. subtilis* cell septation leads to the formation of a high curvature region, energetically suitable for chemoreceptor trimers, while in *E. coli* the cell constriction during division results in only moderate curvature of the membrane (Strahl *et al.* 2015). In this case thus receptors would not have a “preference” for the division site over the cell poles or the lateral membrane.

Despite the different localization mechanisms, the localization of Che proteins primarily to the poles might have a common biological significance in *E. coli* and *B. subtilis*, ensuring that each daughter cell inherits a Che cluster after division. In *Bacillus*, having a cluster at the division site ensures that the new poles of the daughter cells inherit a small cluster (Figure 5B) while dividing *E. coli* has at least two clusters, one at each pole, ensuring that each daughter cell inherits a cluster by inheriting an old cell pole (Figure 6B).

Molecular determinants of receptor polar localization and segregation mechanisms in *Vibrio* spp

While in *E. coli* and *B. subtilis* the polar targeting of bacterial chemoreceptors is due to intrinsic properties of these proteins, it has been recently shown that in *Vibrio cholerae* and *Vibrio parahaemolyticus* the Che proteins are recruited to the cell poles by a set of specialized proteins responsible of the general maturation of these cell regions.

A central determinant of the pole maturation in *Vibrio* is HubP. HubP localizes at one cell pole and is targeted to the future old pole of the daughter cell prior to cell division. The mechanisms behind the targeting of HubP to the cell poles are not understood, but an N-terminal protein region containing a LysM domain might be important to anchor HubP to the peptidoglycan.

Once at the cell pole, HubP recruits a series of ParA-like proteins to this location, which are important for the localization of the origin of replication of chromosome I (ParA), the flagellum (FlhG) and the Che II system (ParC).

ParA proteins were discovered as part of a tripartite system used to actively segregate low copy number plasmids (Austin and Abeles 1983). The other parts of the system are a second protein, ParB, and a specific DNA sequence, *parS*. Three distinct phylogenetic groups of ParA proteins have been described (Ringgaard *et al.* 2011). ParAs of group one are encoded by the chromosome and are mostly involved in chromosome segregation; the second group includes ParA proteins mostly plasmid-encoded and important in plasmid segregation. Finally, a third class, which has been identified more recently, comprises ParA-like proteins encoded by gene clusters also encoding chemotaxis proteins (Ringgaard *et al.* 2011). Groups one and two share a number of functional features. When bound to ATP, ParA is able to dimerize and the ATP bound dimer is able to bind to the nucleoid. ParB specifically binds to the *parS* sequence forming the partition complex. The low basal ATPase activity is stimulated via interaction with the partition complex. The ADP bound form of ParA monomerizes and is unable to bind the nucleoid. As the partition complex interacts with and releases ParA from the nucleoid it will move in the direction of the highest concentration of bound ParA i.e. away from the region of the nucleoid, which it has just stripped of ParA (Lutkenhaus 2012).

Less known are the ParA-like proteins belonging to the third group. These ParA-like proteins do not have an obvious cognate ParB. One example is ParC, encoded by the main chemotaxis operon of *Vibrio cholerae* (Cluster II) (Ringgaard *et al.* 2011). As briefly mention above, ParC localizes at one cell pole at the beginning of the cell cycle in a HubP-dependent manner. At this same location, the CheW belonging to the main chemosensory system also forms a cluster. Then, as the cells elongate, a new ParC focus is formed at the other cell pole and is immediately followed by a second CheW cluster (Figure 7A). Later, when the cell divides into two daughter cells, each will inherit a pole and thus a ParC focus as well as a Che cluster. In the absence of ParC, the Che clusters are randomly positioned, suggesting that not only does ParC synchronize the maturation of the cell pole with the proceeding of the cell cycle, but also it specifically targets Che proteins at this location. In some cells lacking ParC, the Che cluster localizes to the cell pole, suggesting that other factors might intervene in the polar targeting of the chemosensory system in *Vibrio*.

ParC might mediate the polar targeting of Che proteins by the intermediate of ParP (Ringgaard *et al.* 2014) (Figure 7B). ParP is encoded by a gene immediately downstream of *parC* and it contains a CheW-like domain as well as a proline rich region that has been shown to interact

with the localization and inheritance domain (LID) CheA and ParC. LID is only present in CheA that are coexpressed with ParC and ParP. In cells depleted of ParP, Che arrays show an impaired localization very similar to that observed in cells lacking ParC. The polar localization of ParP depends on ParC and the two proteins are recruited at the pole at the same time and before CheW. Fluorescence recovery photobleaching experiments (FRAP) suggest that ParP stabilizes CheA in the chemosensory clusters (Ringgaard *et al.* 2014).

The ParC-mediated localization of *Vibrio* Che proteins is an active mechanism, as it requires ATP hydrolysis by ParC. Therefore, it differs from the *E. coli* and *B. subtilis* localization mechanisms that are not energy consuming. It is not known how HubP recruits ParC at the cell pole as these two proteins have been shown not to directly interact. On the other hand, HubP directly interacts with the other ParA homologs such as ParAI and FlhG enabling targeting at the cell pole. Beside facilitating its partitioning upon cell division, it has been proposed that the biological significance of targeting the Che cluster at the pole in *Vibrio* is to associate it spatially with the polar flagellum. Such colocalization would minimize the requirement for diffusion of phosphorylated CheY between the two systems and, thus, produce a more rapid response to external signals. The diffusion rate of CheY in *E. coli* is not thought to limit signaling, but possible the effect of positioning a Che cluster at the opposite pole to the flagellum on response kinetics has not been tested.

The *R. sphaeroides* cytoplasmic chemosensory system and its ParA-like mediated partitioning

As previously mentioned *R. sphaeroides* has two sets of chemotaxis proteins organized into spatially separate arrays: one transmembrane and polar, the other soluble and cytoplasmic (Wadhams *et al.* 2003). At the beginning of the cell cycle cells possess a single cytoplasmic cluster, which is broadly centered about the mid-cell (Figure 7D and E). Prior to cell division the cell forms a second cluster, the two arrays are positioned at $\frac{1}{4}$ and $\frac{3}{4}$ positions along the cell length (Thompson, Wadhams and Armitage 2006) (Figure 7D and E). It has been shown that this dynamic localization pattern ensures that when the cells divide both daughter cells inherit a complete set of chemotaxis proteins (Figure 7D and E). The positioning pattern and segregation of the cytoplasmic cluster of *R. sphaeroides* is dependent on a ParA-like protein, PpfA, whose gene is encoded in the same operon as the majority of the components of the

cluster (Thompson, Wadhams and Armitage 2006). The *ppfA* gene is immediately upstream of *tlpT*, the gene for major of the two soluble chemoreceptors. Deletion of *ppfA* results in a loss of duplication of the cluster with cells never having more than one cluster (Roberts *et al.* 2012). On division, one daughter cell will not inherit any of the cytoplasmic chemosensory proteins and has to synthesize them *de novo*, rendering it non-chemotactic during this time. This phenotype is also seen when the N-terminal 120 amino acids of TlpT are removed, suggesting that the PpfA links the cluster to the nucleoid via this region of TlpT (Roberts *et al.* 2012).

Interestingly PpfA does not belong to the third phylogenetic group including ParA-like encoded by operons containing chemotaxis genes. It is, in fact, phylogenetically closer to ParA-like whose genes are on plasmids. It is therefore possible that PpfA controls the equal partitioning of the *R. sphaeroides* cytoplasmic Che clusters through mechanisms similar to those proposed for the ParA-mediated plasmid segregation (Ringgaard *et al.* 2011).

The polar localization of the *R. sphaeroides* transmembrane Che cluster superficially resembles that of *E. coli*. Time lapse analysis of cluster behavior followed using CheW fusions shows that relatively evenly spaced clusters of about 800 receptors diffuse freely in the membrane, slowing slightly at the polar regions, probably accounting for the increased fluorescence observed at the poles. Sphaeroplasts have evenly spaced clusters again suggesting a nucleation and reaction-diffusion mechanisms, as seen in *E. coli*. Interestingly however, clusters appeared to be excluded from regions of division, never seen close to FtsZ rings and therefore very different to the mechanisms suggested for *B. subtilis* (Chiu *et al.* 2013) (Strahl *et al.* 2015). The *R. sphaeroides* cell poles are not perfectly round and appear to contain a slightly protruding division scar. Such shape would generate a small region of high curvature that could accommodate the transmembrane receptor trimers or dimers (Tucker *et al.* 2010; Strahl *et al.* 2015).

The nucleoid-dependent localization and segregation of the cytoplasmic *M. xanthus* Frz pathway

While the cytoplasmic TlpT receptor and associated chemotaxis proteins of *R. sphaeroides* form a single array at the center of cells, the FrzCD cytoplasmic chemoreceptor of the *M. xanthus* Frz pathway localizes in multiple clusters distributed along the cell body (Mauriello *et al.* 2009; Moine *et al.* 2014) (Moine 2017). Interestingly, FrzCD clusters do not occupy the whole cell length but only the central region where they colocalize with the nucleoid (Figure 8A). Once associated with the nucleoid, FrzCD recruits FrzE (CheA) (Moine 2017) and, probably, FrzA (CheW) to form active signaling units. The FrzCD colocalization with the nucleoid is because this protein can directly interact with DNA in a DNA-sequence independent manner and through its N-terminal domain, which contains a highly positively charged peptide. This peptide distantly resembles an amino acid tail present at the N terminus of eukaryotic histones, important for the correct assembly of the nucleosomes (Parra *et al.* 2006; Iwasaki *et al.* 2013). The lack of the FrzCD N-terminus causes the loss of the association with the nucleoid and the dispersal of FrzCD in the cytoplasm, suggesting that the FrzCD-DNA binding is strictly required for cluster formation. Like other bacteria, cluster formation also requires the presence of the CheA-like FrzE.

Several lines of evidence suggest that the Frz cluster formation on the nucleoid occurs in a stochastic manner similarly to the assembly of the *E. coli* Che lattices in the membrane. First, the initial binding of FrzCD to DNA might take place anywhere on the nucleoid as such binding is not DNA-sequence specific. Once recruited to the nucleoid, FrzCD molecules nucleate clusters that move on the nucleoid surface, however exploring only confined small areas. These areas might represent the minimal critical distance from other clusters at which foci can exist (Moine 2017). In other words, FrzCD molecules can either nucleate new dynamic foci if they are far enough from existing clusters, or encounter and join a neighboring cluster.

Thus, the *M. xanthus* cytoplasmic Frz system might form signaling clusters on the bacterial chromosome using mechanisms similar to those of transmembrane chemoreceptors using bacterial inner membrane as a platform to form the arrays of trimers of dimers (Briegel *et al.*, 2012). One outcome of this nucleoid-driven cluster assembly could be to ensure that the cytoplasmic receptors have the same properties as transmembrane receptors but in response to intracellular signals. One of these properties is the sensitivity to a small percentage change over a wide range of effector concentration. Indeed, it has been shown that while cells with nucleoid-anchored chemoreceptor clusters are very sensitive to a wide range of a known Frz

activator, cells carrying a receptor mutant that is no longer capable of binding to the nucleoid and forming clusters can only respond to limited signal concentrations (Moine 2017).

Why FrzCD needs to form many rather than just one single cluster is unclear. One possibility is that it prevents CheY-P diffusion to the polar and lateral motors being limiting, given the length of *M. xanthus* cells (5-10 μm in average). However, a more attractive explanation is that the nucleoid-dependent formation of multiple distributed clusters represents a simple mechanism to segregate the clusters during cell division without the need for a faithful partitioning system, as is required for the a single cluster in *R. sphaeroides*.

Beside the Frz pathway, the Dif, Che4, Che5 and Che6 systems form transmembrane distributed clusters whereas Che7 forms polar clusters. These clusters are all very dynamic and the determinants of their localization patterns have yet to be discovered (Moine *et al.* 2014).

CONCLUDING REMARKS

How does the number and positioning of MCP clusters relate to bacterial cell behaviors? It has been proposed that one or two chemosensory clusters in swimming bacteria that are approximately 2 μm long, are sufficient to respond to temporal gradients with maximum efficiency and that the regulation of the activity of one or multiple peritrichous flagella is not limited by the CheY-P diffusion (Berg and Purcell 1977). Other bacteria, such as *M. xanthus* and *P. aeruginosa*, possess multiple chemosensory clusters. Such bacteria move on solid surfaces and may be longer than *E. coli*. or *R. sphaeroides*. Thus, the presence of multiple distributed Che clusters might have two functions: the first function ensure that the activity of the polar TFP (for both *M. xanthus* and *P. aeruginosa*) and FAC (for *M. xanthus*) is not affected by the CheY-P diffusion as such molecule would be produces at multiple sites within cells. Another function could be that bacteria gliding on non-homogenous solid surfaces and continuously making contacts with the surface as well as with neighboring cells (of the same or different species), might need to perceive the environment by both spatial and temporal sensing mechanisms.

In *R. sphaeroides* the presence of two chemosensory clusters with different receptor composition and distantly positioned, one at the cell pole and one within the cytoplasm, most probably allows the cells to sense both the external environment and intracellular signals,

reflecting the metabolic state. Similarly, the different localization patterns of the various *M. xanthus* CSS might serve to separate the sensing of specific sets of signals at different cell regions. In both *R. sphaeroides* and *M. xanthus*, protein specificity allow that each different Che cluster to recruits the correct set of proteins (Scott *et al.* 2010; Moine *et al.* 2014).

As discussed above, the CSS subcellular localization functions to ensure the inheritance of Che clusters within the bacterial population, by guaranteeing that each daughter cell inherits both polar and cytoplasmic clusters upon division. In some bacterial species a second Che cluster is produced before the beginning of cell division. Interestingly, the formation of this second cluster as well as its segregation is driven by different mechanisms in different bacteria. For example, in *R. sphaeroides* the formation of an additional Che cluster soon before the beginning of cell division is mediated by a ParA-like protein, which also actively transports and segregates the two resulting clusters in the future daughter cells probably by mechanisms similar to those described for plasmid segregation. In *Vibrio spp.*, before the beginning of cell division, a second Che cluster forms and is stabilized at the opposite cell pole by the intermediate of a ParA-like protein. However, in this case, the recruitment of the second cluster at the opposite pole is not driven by the active transport by ParA, but rather by protein recognition. In fact, before cell division, first a second ParA focus forms at the opposite cell pole. Then MCP molecules recognize and bind ParA, nucleating a new cluster at this site. The inheritance of the two Che clusters will occur along with that of the cell poles.

In *B. subtilis* the formation of a second Che cluster occurs after cell division has started when a highly curved regions at the pre-division site forms, which is recognized by the MCPs.

While the faithful segregation of the CSS might represent a role in most bacteria, the nucleoid-driven localization of the *M. xanthus* Frz system might represent an exception as the presence of multiple distributed clusters on the nucleoid might bypass the need for a faithful segregation mechanism.

Add

Chiu, S-W, Roberts, MAJ, Leake, MC and Armitage, JP (2013) Positioning of FtsZ and chemosensory proteins through the *Rhodobacter sphaeroides* cell cycle *MolMicrobiol* 90: 322-327

Also the *S. meliloti* CheY phosphate sink

The bibliography isn't consistent in style-there are caps in some titles, and no species italicised

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Figure legend

Figure 1. (A) *E. coli* smooth swimming occurs when helical flagella rotate in the counterclockwise (CCW) direction and form a bundle. A switch in the direction of flagellar

rotation from CCW to clockwise to clockwise (CW) causes a tumble that reorients the bacterium. While the tumbling frequency is constant in homogeneous environments (green box on the left), it is biased in gradients of effector molecules (green gradient). **(B and C)** Schematic representation of the *E. coli* (B) and *B. subtilis* Che systems (C).

Figure 2. (A) Schematic representation of the *R. sphaeroides* transmembrane and cytoplasmic chemosensory clusters. **(B)** *R. sphaeroides* linear swimming occurs when the single flagellum rotates in the CW direction. A stop in the flagellar rotation causes a reorientation of the bacteria. Analogously to *E. coli*, while the frequency of stops is constant in homogeneous environments, it is biased in gradients of molecules.

Figure 3. (A) *M. xanthus* cells move forward by the aid of two motility systems: a Type Four Pilus (TFP)-mediated motility where pili extend, bind the exopolysaccharide of a neighboring cells and retract pulling the first cell forward; a Focal Adhesion Complex (FAC)-mediated motility where multiprotein trans-wall complexes, energized by the proton motive force, are assembled at the leading cell pole (red circle) and transported along the cell (colored circles) on an internal yet unidentified rail. When FAC encounter the substratum, they bind on it, thus remaining fixed relative to the substratum and exerting the force necessary to push the cell forward. Cells reverse the direction of their movement at varying frequencies depending on the presence of inhibitors or activators in the medium. It has been proposed that the *M. xanthus* reversals allow the bacteria to reorient themselves in the environment analogously to the *E. coli* tumbling and the *R. sphearoides* flagellar stops. **(B)** Schematic representation of the *M. xanthus* eight chemosensory systems and their functions.

Figure 4. (A) MCP form trimers of dimers (each dimer is shown as a green circle) organized in hexagonal arrays together with CheA (dark blue bars) and CheW (white bars) rings. A signaling unit is represented in the red box. **(B)** The constituents of a signaling unit. **(C-D)** Cryoelectrotomography images from Briegel et al., 2014 of transmembrane (C) and cytoplasmic (D) MCP lattices (left panels) and their schematic representations (right panels; color codes as in (A)).

Figure 6 Largest chemotaxis clusters are found at the poles, small lateral clusters are found in all cells. DIC images (inset) correspond to cell outlines (dashed lines). (A and B) Two representative *Δtar* cells with pALM6001 (Tar-mEos).

Figure 5. (A) The *B. subtilis* TlpA chemoreceptor localizes at the cell poles and at the division site (scale bar, 3 μm; image adapted from Strahl et al., 2015). **(B)** The TlpA (green) polar localization results from the recognition of highly curved zones at the division site, which correspond to the future poles in the daughter cells after division. **(C)** Schematic representation of a TlpA trimer of dimer inserted in a curved membrane region and the consequences of specific point mutations (image adapted from Strahl et al., 2015).

Figure 6. (A) Tar-mEos localization by PALM microscopy (image adapted from Greenfield et al., 2009). **(B)** Schematic representation of the stochastic self assembly model proposed by Thiem and Sourjik (Thiem and Sourjik, 2008) for the *E. coli* MCP cluster formation .

Figure 7. (A) Time-lapse of the *V. cholerae* YFP-CheW1 polar localization showing the appearance of a second Che cluster at the opposite cell pole after cell division (image adapted from Ringaard et al., 2011). **(B)** Schematic representation of the molecular determinants behind the polar localization of the *V. cholerae* Che system. **(C)** Time-lapse of the *R. sphaeroides* TlpT-YFP cytoplasmic chemoreceptor. One cell containing two clusters divides into cells A and B. The single A cluster becomes two clusters positioned at 1/4 and 3/4 of the cell length. This positioning results in each daughter cell inheriting a cluster at about mid-cell (image adapted from Thompson et al., 2006). **(D)** Schematic representation of the duplication and segregation of the *R. sphaeroides* cytoplasmic chemosensory cluster (green circles).

Figure 8. (A) *M. xanthus* FrzCD-GFP localization at the nucleoid. The nucleoid is visualized with the DNA-DAPI staining. **(B)** A model showing the stochastic assemble of Frz chemosensory clusters (green) on the nucleoid (blue) and their uneven segregation upon cell division.