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Review Article

REVIEW ARTICLE

Cellular targeting and segregation of bacterial chemosensory systems

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One sentence summary: The authors summarize the current understanding of the architecture and the segregation mechanisms of chemoreceptors in few bacterial model systems, *Escherichia coli*, *Bacillus subtilis*, *Rhodobacter sphaeroides*. *Vibrio spp* and *Myxococcus xanthus*.
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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 chemoreceptor 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 and suggest that the pattern of chemoreceptor distribution is coupled to behavioral life-style of that species.

Keywords: chemotaxis; localization; partitioning; bacterial receptors; signal transduction; MCP

INTRODUCTION

The ability of all organisms to perceive changes in the environment and adapt their behavior in response is an 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 (Groisman 2016). Chemotaxis or chemosensory systems (CSS) are modified two-component systems that during evolution acquired accessory modules (Wuichet and Zhulin 2010), conferring new functions that increased the range of responses and sensitivity of the system (Bray, Levin and Morton-Firth 1998; Sourjik and Berg 2002). CSS can perceive multiple signals thanks to the presence of multiple specialized receptors (Bi and Sourjik 2018). The organization of these receptors appears

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to be common across species (Briegel *et al.* 2009, 2015) and allows receptor cooperativity and the amplification of the initial signal (Li and Hazelbauer 2014; Frank *et al.* 2016; Piñas *et al.* 2016). 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 (Yuan *et al.* 2012). Beside motility, CSS can modulate a large variety of functions, such as cyst formation (Berleman and Bauer 2005), biofilm formation (Corral-Lugo *et al.* 2016), 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 that look like intricate honeycomb-like lattices when examined using cryo-electron microscopy (Briegel *et al.* 2008, 2012; Khursigara, Wu and Subramaniam 2008; 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 chemosensory proteins 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 (Alley, Maddock and Shapiro 1992; Sourjik and Berg 2000; Wadhams *et al.* 2002; Bardy and Maddock 2005; Ringgaard *et al.* 2011; Moine *et al.* 2014; Strahl *et al.* 2015). How are CSS targeted to their final cell location and how are they segregated between daughter cells? In this manuscript, we will review some recent findings on the cellular positioning of chemoreceptors in well-characterized bacterial model systems, such as *Escherichia coli*, *Bacillus subtilis*, *Rhodobacter sphaeroides, Vibrio spp* 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* CSS: composition and signal transduction

E. coli, like many bacteria, moves through its environment using the rotation of multiple helical flagella (typically 4–6) (Leifson 1960; Schuhmacher, Thormann and Bange 2015). When swimming, the flagella all rotate in a counter-clockwise (CCW) direction coming together in a bundle (Fig. 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 (Brown, Delalez and Armitage 2011). 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) (Fig. 1A). In *E.* coli, 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 (Darnton *et al.* 2007) (Fig. 1A). When all flagella resume CCW rotation, the bundle reforms and the cell swims smoothly in a new direction. This behavior is termed chemotaxis.

This switch in direction of flagellar rotation in response to changing environmental conditions is controlled by a CSS (Fig. 1B). Transmembrane receptors, known as chemoreceptors or methyl-accepting chemotaxis proteins (MCPs), detect the presence of attractants and repellents via sensing domains in the periplasm (Neumann *et al.* 2010; Ortega, Zhulin and Krell 2017). Binding of these effectors results in a conformational change, which is transmitted through the inner membrane to the cytoplasmic tip of the chemoreceptor (Falke and Erbse 2009; Ames, Hunter and Parkinson 2016). The conformational state of the chemoreceptor controls the activity of a histidine kinase, CheA, which binds to the cytoplasmic tip of the chemoreceptors, along with an adaptor protein CheW. When conditions are improving, i.e. increasing attractants or decreasing repellents, the chemoreceptors are in a state that inhibits the kinase activity of CheA. The converse is true when conditions are worsening, resulting in high CheA activity (Bi and Sourjik 2018). 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 (Bi and Sourjik 2018). 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 (Fig. 1B) (Vaknin and Berg 2004). CheZ rapidly dephosphorylates CheY-P, terminating the signal and preventing the cell from tumbling for a prolonged amount of time (Fig. 1B) (Bren *et al.* 1996; Yuan *et al.* 2012).

To reset the signaling state, specific glutamate residues on the chemoreceptors are demethylated by the phosphorylated form of CheB. This decreases the sensitivity of the system by decreasing the chemoreceptor 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 (Sourjik and Berg 2002; Clausznitzer *et al.* 2010; Bi and Sourjik 2018).

While *E. coli* represents the paradigm for the study of CSS in Gram-negative bacteria, *B. subtilis* is the paradigm for Gram-positive bacteria. Like *E. coli, B. subtilis* also swims using peritrichous flagella. While the flagellar activity is modulated by a CSS as in *E. coli,* 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 (Rao and Ordal 2009) (Fig. 1C).

Differently from the methylation system regulating *E. coli* chemotaxis, chemoreceptor methylation in *B. subtilis* regulates receptor-kinase activity in a site-specific manner (Zimmer *et al.* 2000; Glekas *et al.* 2011).

While CheZ is absent in the *B. subtlis* CSS, indeed in all non-enteric species, two additional adaptation modules are present: CheC-CheD and CheV (Fig. 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 (Glekas *et al.* 2012). CheD also forms a ternary complex with CheC and CheY-P where it induces the dephosphorylation of CheY-P by CheC, thus increasing tumbling (Muff and Ordal 2007). CheV is a fusion between a CheW and a response regulator domain (Rao, Glekas and Ordal 2008). It has been proposed that this protein functions depending on its phosphorylation state and on the receptor methylation pattern (Walukiewicz *et al.* 2014).

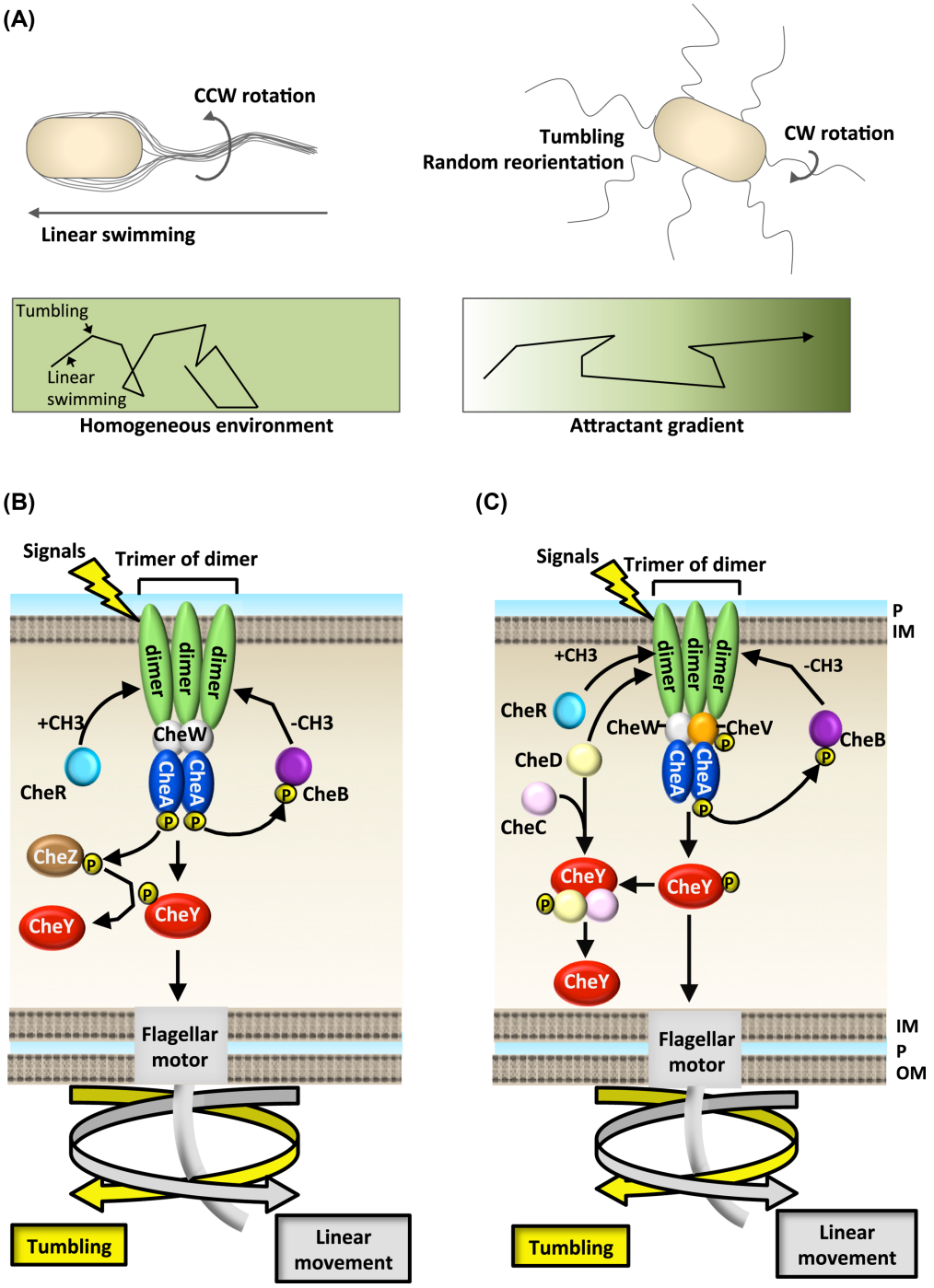

Figure 1. (**A**) *E. coli* smooth swimming occurs when helical flagella rotate in the counterclockwise (CCW) direction and form a bundle. A switch in the direction of flagellar rotation from counterclockwise (CCW) to clockwise (CW) causes a tumble that reorients the bacterium. While the tumbling frequency is constant in homogeneous environments (green box on the left), it is biased in gradients of effector molecules (green gradient). (**B**) Schematic representation of the *E. coli* chemosensory system. Transmembrane receptor trimers of dimers (green) sense signals into the periplasmic space. If the signal consists into an attractant, CheA (blue) phosphorylation is inhibited, CheY (red) remains into its unphosphorylated state and there is no change in the flagellar rotation. The result is that bacteria do no change their swimming direction (linear swimming in (A)). Conversely, the binding of receptors to a negative signal (repellent) induces CheA phosphorylation, the increase of CheY-P and flagellar tumbling. Tumbling ultimately allows the reorientation of the bacterial cells. CheR (light blue) and CheB (purple) reset the system to a prestimulus state allowing adaptation. CheB is active when phosphorylated by CheA. For example, in the presence of a persistent negative stimulus, CheA-P phosphorylates CheB (and CheY). CheB-P demethylates active chemoreceptors thus reducing their ability to activate CheA. This process decreases the concentration of CheY-P and resets the system. Conversely, in the presence of a persistent positive stimulus, CheA-P is reduced and so are CheY-P and CheB-P. This favors chemoreceptors methylation, which in turn increases CheA-P again resetting the system to the equilibrium. CheZ (brown) is involved in signal termination by dephosphorylating CheY. (**C**) Unlike E. coli, in *B. subtilis*, attractants induce the accumulation CheY-P, which in turn reduces upon binding to the flagellar motor, instead of increases, tumbling. In complex with CheD (light yellow) and CheY-P, CheC (pink) dephosphorylates CheY-P thus increasing tumbling. CheV (orange) is a fusion between a CheW and a response regulator domain that can modulate CheA activity depending on the receptor methylation pattern.

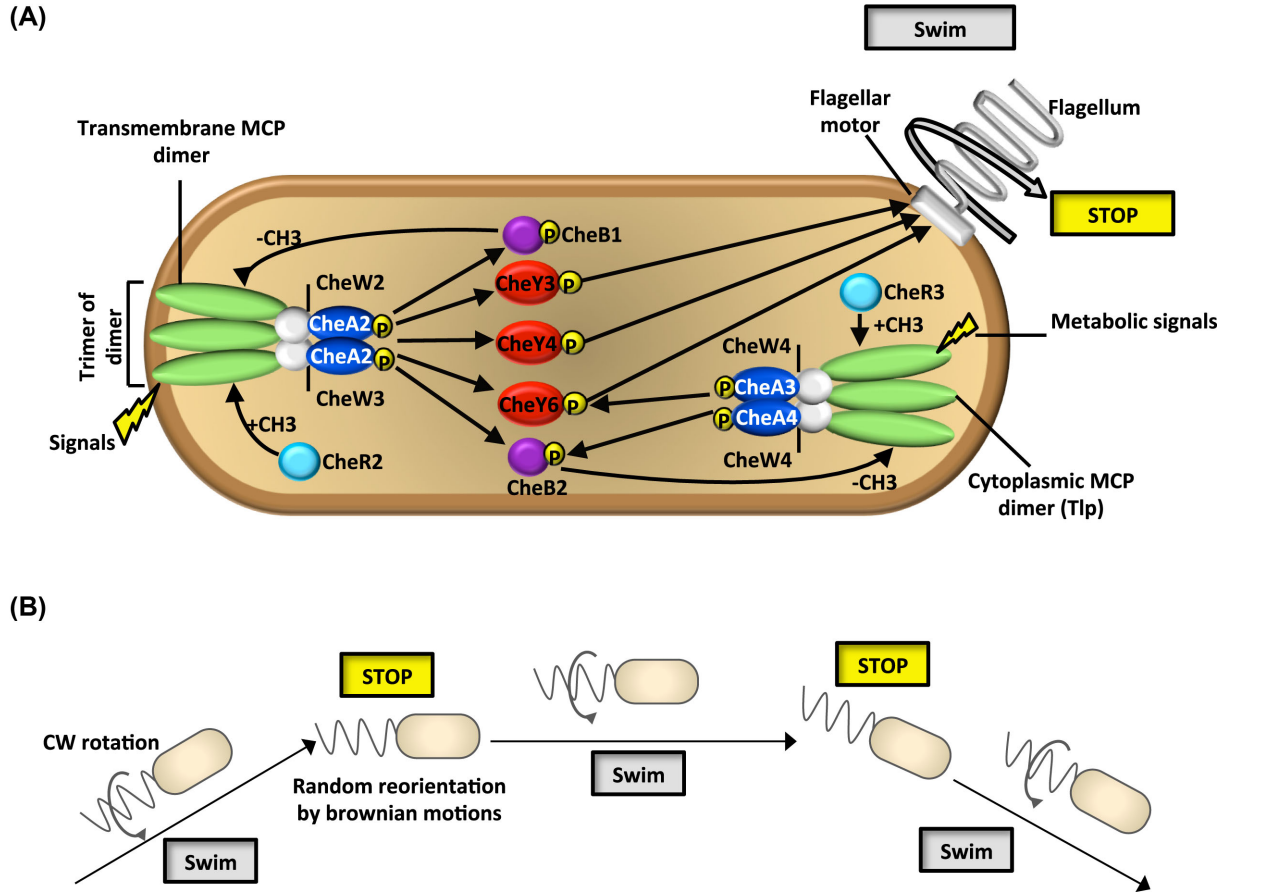

Figure 2. (**A**) Schematic representation of the *R. sphaeroides* transmembrane and cytoplasmic chemosensory clusters. While the transmembrane chemoreceptors modulate the phosphorylation of CheA2, cytoplasmic Tlp induces the accumulation of CheA3-P and CheA4-P. CheA2-P transfers phosphoryl groups to CheY3, cheY4 and CheY6. These three response regulators, in their phospshorylated state, stimulate flagellar stops and the reorientation of the bacterial cell as shown in (B). CheY6 can be phosphorylated also by CheA3 and CheA4 thus acting as a central signaling output. The phosphorylation reactions involving CheB2-P connect the polar and cytoplasmic 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.

Rhodobacter sphaeroides: keeping the outside in tune with the inside

Unlike *E. coli*, which has a single copy of each of the chemosensory genes (excluding the chemoreceptor encoding genes) the *Rhodobacter sphaeroides* genome encodes 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 homologues to chemosensory proteins (Hamer *et al.* 2010; Wuichet and Zhulin 2010; Collins, Lacal and Ottemann 2014).

In *R. sphaeroides*, the majority of these chemosensory genes are organized in three operons. Only the deletion of operon 2 and 3 significantly affects chemotaxis under normal laboratory conditions (Hamblin *et al.* 1997; Porter *et al.* 2002). The products of the two main operons form spatially distinct signaling pathways, one localizes with the transmembrane chemoreceptors, very much like *E. coli*, while the other localizes to the cytoplasm with soluble chemoreceptors (Martin, Wadhams and Armitage 2001; Wadhams *et al.* 2002; Briegel *et al.* 2014) (Fig. 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 CheA3 and 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) (Fig. 2A). *Rhodobacter 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* (Amin *et al.* 2014). *Rhodobacter sphaeroides* has a single, randomly positioned, flagellum that rotates CCW to push the cell and, rather than switching rotational direction stops periodically. Brownian motion reorients the cells during stops, ensuring that the cell is swimming in a new direction when it starts rotating again (Armitage *et al.* 1999; Pilizota *et al.* 2009) (Fig. 2B). The need for two CSS both feeding into a single motor is still

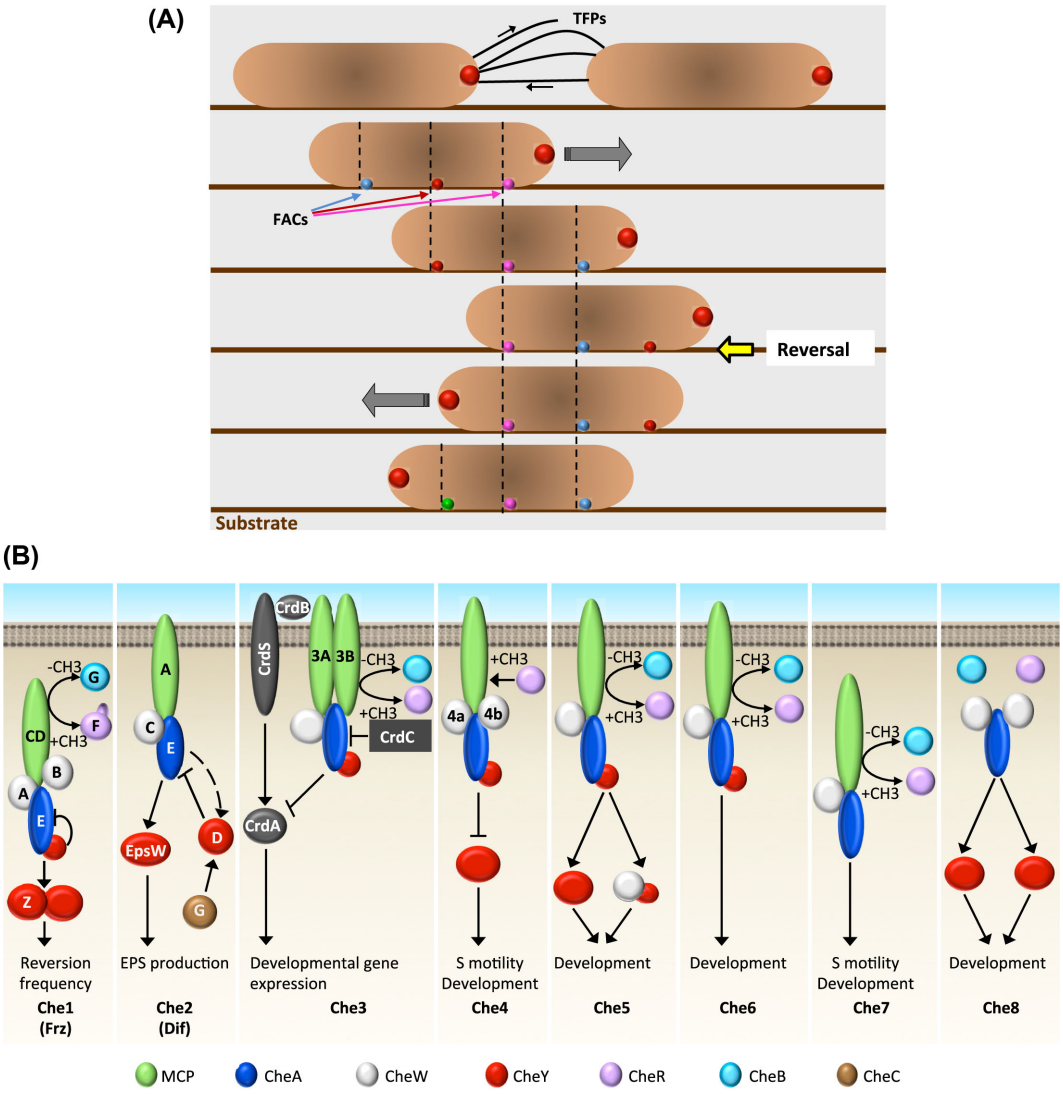

Figure 3. (**A**) *M. xanthus* cells move forward by the aid of two motility systems: a Type Four Pilus (TFP)-mediated motility where pili extend, bind the exopolysaccharide of a neighboring cells and retract pulling the first cell forward; a Focal Adhesion Complex (FAC)-mediated motility where multiprotein 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.

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.

Like *R. sphaeroides*, *Vibrio cholerae* also possesses three gene clusters encoding CSS (Wuichet and Zhulin 2010). Only cluster II has been shown to be important during chemotaxis under laboratory conditions (Gosink *et al.* 2002; Hyakutake *et al.* 2005).

Myxococcus xanthus: modular organization of multiple CSS

More extreme is *M. xanthus* whose genome contains even more genes encoding chemosensory proteins. *M. xanthus* 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 that also requires the production of exopolysaccharide (EPS) (Black, Xu and Yang 2006; Chang *et al.* 2016); and a second motility system that uses internal focal adhesion complexes (FAC) to power motion (Faure *et al.* 2016) (Fig. 3A). In addition to eight operons containing complete sets of CSS encoding genes, *M. xanthus* also encodes 21 chemoreceptors and several orphan chemosensory proteins (Blackhart and Zusman 1985; Yang *et al.* 2000; Kirby and Zusman 2003; Vlamakis, Kirby and Zusman 2004; Moine *et al.* 2014). Frz (Che1) and Che7 are cytoplasmic CSS (Darnell *et al.* 2014; Moine *et al.* 2014, 2017). 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

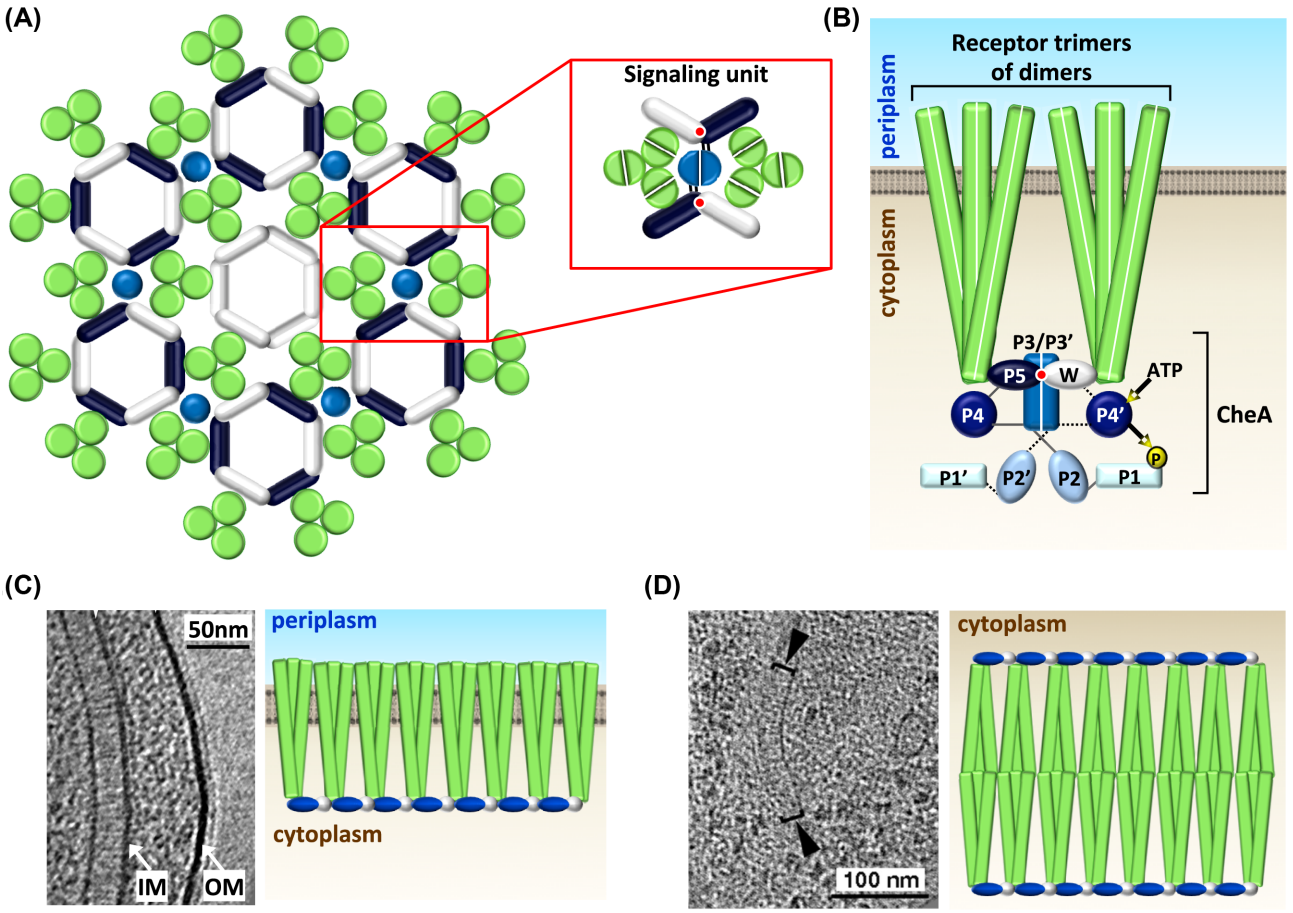

Figure 4. (**A**) MCP form trimers of dimers (each dimer is shown as a green circle) organized in hexagonal arrays together with CheA (dark blue bars) and CheW (white bars) rings. A signaling unit is represented in the red box. The figure has been adapted from Guiseppi *et al.* 2017. (**B**) The constituents of a signaling unit. (**C-D**) Cryoelectro-tomography 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)).

Che4-Che8, Che3 is known to regulate the expression of genes important for fruiting body formation (Kirby and Zusman 2003; Willett and Kirby 2013). The Dif (Che2) system is involved in the activation of EPS production, thus having an essential function in the TFP-mediated motility (Black, Xu and Yang 2006; Black *et al.* 2010). 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* (Bustamante *et al.* 2004; Guzzo *et al.* 2015) (Fig. 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 CHEMOSENSORY PROTEINS ARE ORGANIZED IN HIGHLY ORDERED STRUCTURES

The unique properties of CSS result from their universal macromolecular architecture. Using high-resolution cryoelectron microscopy, CSS appear organized in an intricate 2D honey comb-like structure in which hexagonal shapes are regularly placed with a 12 nm packing distance (Briegel *et al.* 2008; Khursigara, Wu and Subramaniam 2008). The hexagons are made of six chemoreceptor trimers-of-dimers networked by CheA-P5 domain/CheW rings (Fig. 4A), the minimal functional unit consisting in two chemoreceptor trimers-of-dimers, a CheA dimer and two CheW (Fig. 4A and B). Trimers can contain different chemoreceptor 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 (Fig. 4A), but whether these have a biological function is yet to be determined (Briegel *et al.* 2012; Liu *et al.* 2012; Cassidy *et al.* 2015).

This architecture is not strictly required for signal transduction, as one functional unit is enough to generate phosphorylated CheY. However, CSS 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 chemoreceptors into a single array also aids the balancing and integration of signals from multiple signals (Studdert and Parkinson 2004).

The hexagonal organization is common to both transmembrane and cytoplasmic chemoreceptors from Archaea and Bacteria, with some small differences. The transverse view of transmembrane CSS lattices show two plates, parallel and adjacent to the cell membrane, the plate closer to the membrane being composed of chemoreceptors, whereas the distal baseplate of

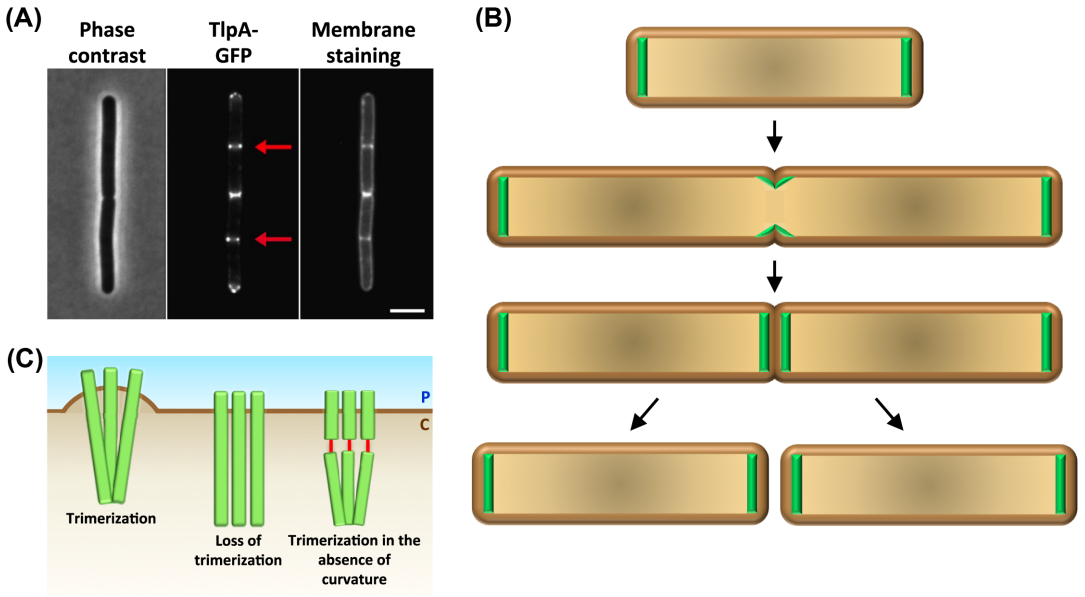

Figure 5. (**A**) The *B. subtilis* TlpA chemoreceptor localizes at the cell poles and at the division site (scale bar, 3 μm; image adapted from Strahl *et al.* 2015). (**B**) The TlpA (green) polar localization results from the recognition of highly curved zones at the division site, which correspond 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).

CheA and CheW (Fig. 4C). Cytoplasmic chemoreceptors 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 (Fig. 4D). Thus, receptors lacking a membrane-binding domain can self-associate through their N-terminal sensing domain or additional stabilizing domains such as that observed in the *V. cholerae* DosM receptor (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. As described below, the labeling of proteins using fluorescent fusions allowed the CSS to be resolved as discrete clusters and their positions and dynamics tracked (Sourjik and Berg 2000; Wadhams *et al.* 2002; Mauriello *et al.* 2009; Ringgaard *et al.* 2011; Strahl *et al.* 2015).

CELLULAR LOCALIZATION AND SEGREGATION OF CHEMOSENSORY ARRAY

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.

Works 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) (Fig. 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 (Fig. 5) (Strahl *et al.* 2015). 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 (Strahl *et al.* 2015). 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 similar to that measured for the base of the cell division septum (Fig. 5C) (Strahl *et al.* 2015). The division site therefore represents an energetically favorable environment for the positioning of TlpA trimers of dimers because of the reduced membrane stress (Endres 2009). Point mutations in TlpA key residues responsible of the formation of chemoreceptor trimers prevent the TlpA accumulation at division plane. Similarly, the insertion of a glycine stretch at the interface between the chemoreceptor 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 (Fig. 5C). These trimers became curvature-insensitive and localized anywhere in the cell membrane (Strahl *et al.* 2015).

While in *B. subtilis* chemoreceptors exclusively localize at the cell poles and at the division sites, the *E. coli* homologues 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) (Fig. 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 (Fig. 6B). Interestingly, by controlling the chemoreceptor expression levels in cells, Thiem and Sourjik

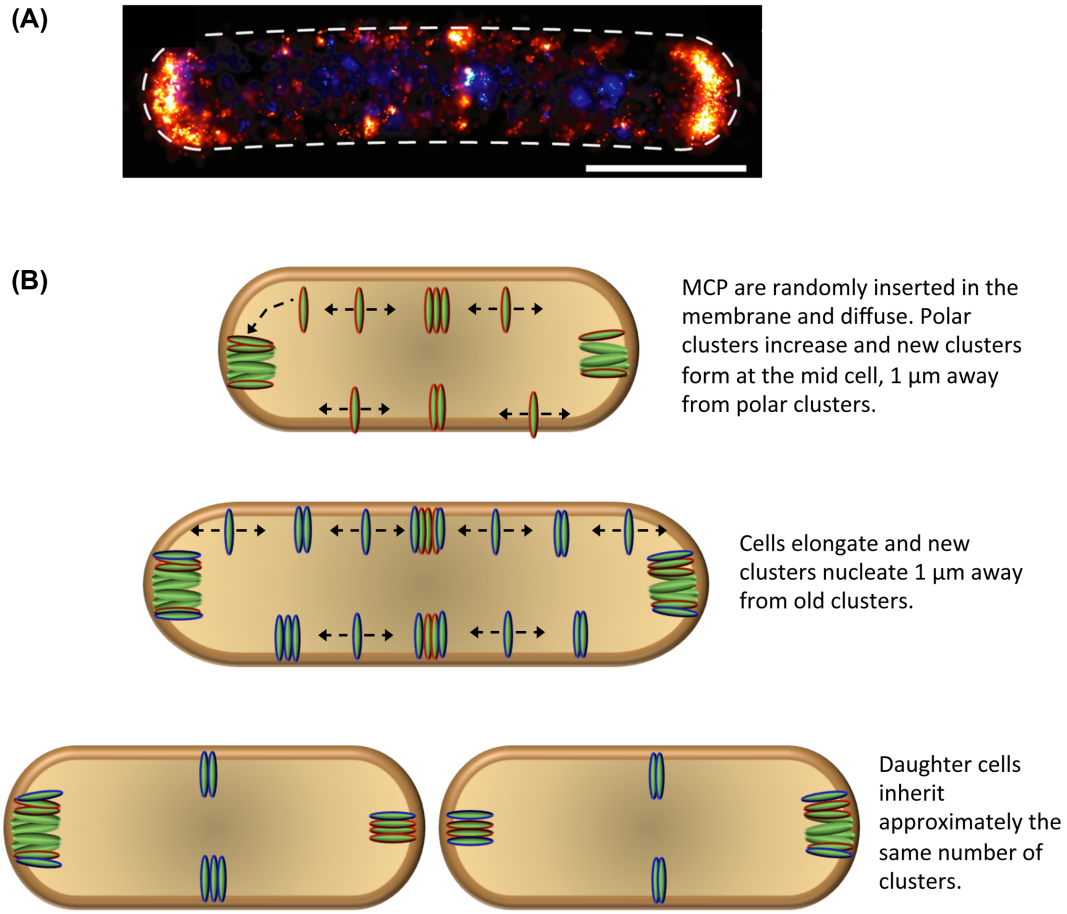

Figure 6. (**A**) Tar-mEos localization by PALM microscopy (image adapted from Greenfield *et al.* 2009). (**B**) Schematic representation of the stochastic self-assembly model proposed by Thiem and Sourjik (2008) for the *E. coli* MCP cluster formation.

(2008) 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. 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 (2008) implies that chemoreceptors 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. 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). Interestingly, it has been recently shown that the transmembrane regions of the high abundance Tar and Tsr *E. coli* chemoreceptors can, alone, mediate the formation of polar and later clusters (Pollard and Sourjik 2018). This intrinsic property of the Tar and Tsr transmembrane domains would explain the ability of these receptors to cluster even in the absence of CheA (Kentner *et al.* 2006; Saaki, Strahl and Hamoen 2018).

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. It has been recently shown that the receptor polar localization might be further stabilized by the effect of the curved membrane (Draper and Liphardt 2017; Saaki, Strahl and Hamoen 2018); the Tol-Pal system (Santos *et al.* 2014; Saaki, Strahl and Hamoen 2018); nucleoid exclusion (Neeli-Venkata *et al.* 2016) or by a preference for the lipid composition of the poles (Mileykovskaya and Dowhan 2000; Santos *et al.* 2014). Finally, the formation of polar clusters might be favored by the cell cycle: 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 (Fig. 6B). 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.

Despite the different localization mechanisms, the localization of chemosensory proteins primarily to the poles might have a common biological significance in *E. coli* and *B. subtilis*, ensuring that each daughter cell inherits a CSS 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 (Fig. 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 (Fig. 6B).

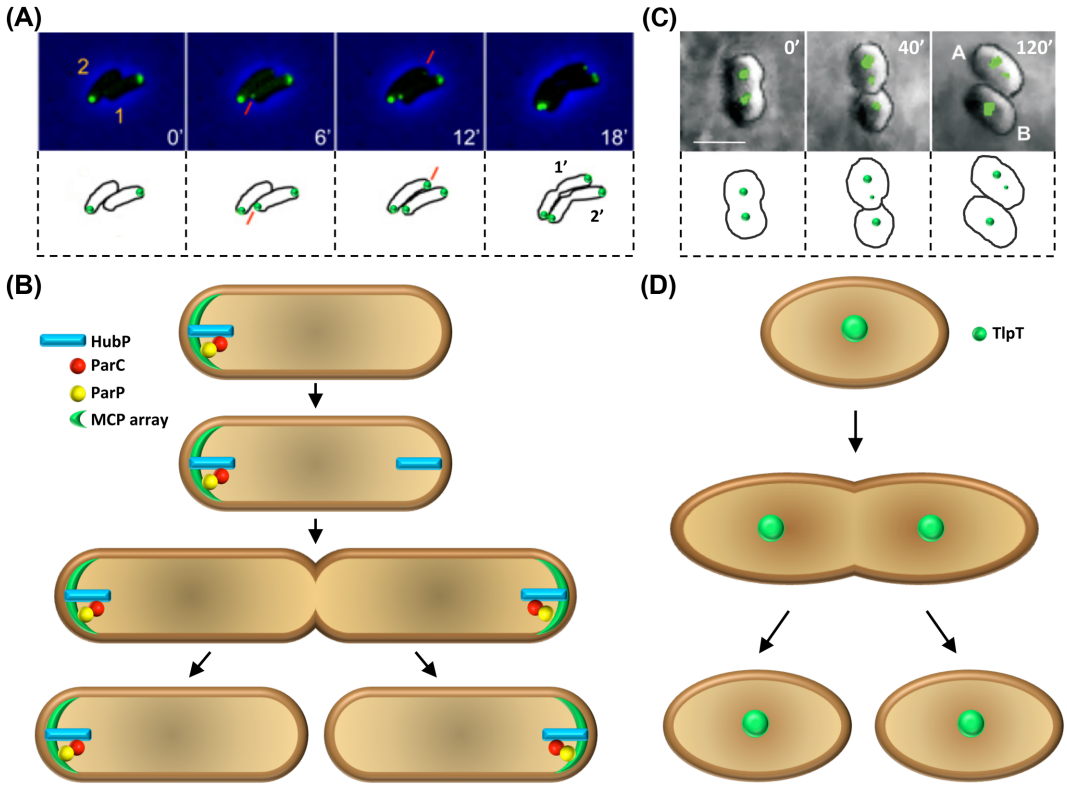

Figure 7. (**A**) Time-lapse of the *V. cholerae* YFP-CheW1 polar localization showing the appearance of a second Che cluster at the opposite cell pole after cell division (image adapted from Ringgaard *et al.* 2011). (**B**) Schematic representation of the molecular determinants behind the polar localization of the *V. cholerae* Che system. The polar hub, HubP (blue), recruits ParC (red) to the flagellated pole. ParC, in turn, recruits ParP (yellow) to this same position. By interacting with the chemoreceptors and CheA, ParP allows the formation of a chemosensory cluster at the flagellated pole. As cells become longer, a second HubP-ParC-ParP cluster form at the opposite pole allowing the formation of a second chemosensory cluster as well. Upon cell division each cell inherits a chemosensory array. (**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, Wadhams and Armitage 2006). (**D**) Schematic representation of the duplication and segregation of the *R. sphaeroides* cytoplasmic chemosensory cluster (green circles).

Molecular determinants of chemoreceptor 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*, chemosensory proteins are recruited to the cell poles by a set of specialized factors responsible of the general maturation of these cell regions.

A central determinant of the pole maturation in *Vibrio* is HubP (Yamaichi *et al.* 2012). HubP localizes at one cell pole and is targeted to the future old pole of the daughter cell prior to cell division (Yamaichi *et al.* 2012; Galli, Paly and Barre 2017). 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 (Yamaichi *et al.* 2012).

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 (ParAI), the flagellum (FlhG) and the Che II system (ParC) (Yamaichi *et al.* 2012). A functional ortholog of *V. cholerae* HubP has been found to be important in the positioning of Che proteins in *Shewanella putrefaciens* (Rossmann *et al.* 2015).

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 consist of 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 chemosensory proteins (Ringgaard *et al.* 2011). These ParA-like proteins do not have an obvious cognate ParB. One example is ParC, encoded together with the Che II system of *Vibrio cholerae* (Ringgaard *et al.* 2011). As briefly mentioned above, ParC localizes at the flagellated cell pole at the beginning of the cell cycle in a HubP-dependent manner. At this same location, the CheW belonging to the main CSS 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, giving rise to a bipolar localization pattern (Fig. 7A). Later, when the cell divides into two daughter cells, each will inherit a pole and thus a ParC focus as well as a CSS cluster. In the absence of ParC, the CSS 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 chemosensory proteins at this location. In some cells lacking ParC, the CSS cluster localizes to the cell pole, suggesting that other factors might intervene in the polar targeting of the chemosensory system in *Vibrio* (Ringgaard *et al.* 2011).

ParC mediates the polar targeting of chemosensory proteins by the intermediate of ParP (Ringgaard *et al.* 2014; Alvarado *et al.* 2017) (Fig. 7B). ParP is encoded by a gene immediately downstream of *parC* and it contains a CheW-like domain, also termed AIF for Array Integration and Formation, as well as a proline rich region. ParP has been shown to interact with the Localization and Inheritance Domain (LID) of *V. cholerae* CheA and with ParC. LID is in between domains P2 and P3 and is only present in CheA that are coexpressed with ParC and ParP (Ringgaard *et al.* 2014). In cells depleted of ParP, CSS arrays show an impaired localization very similar to that observed in cells lacking ParC. The two proteins are recruited at the pole at the same time and before CheW (Ringgaard *et al.* 2014). It has recently been unveiled the role of ParP in CSS cluster formation and localization. First, ParP is recruited at the cell pole via interactions between its N-teminal domain and ParC (Alvarado *et al.* 2017). Then, the two interface AIF domain of ParP interact with the C-terminal tip of the chemoreceptor signaling domain and the P5 domain of CheA, allowing the positioning of these two proteins and their clustering at the cell pole. The binding of CheW to the chemoreceptor and CheA will ultimately allow the formation of complete chemosensory arrays (Alvarado *et al.* 2017). Thus, while in all described CSS the chemoreceptor::CheA::CheW binding is essential for cluster formation, in *V. cholerae* the presence of the newly described multi-interface protein ParP makes the presence of CheA dispensable for cluster formation. Indeed, the presence of chemoreceptors, CheW and either CheA or ParP is sufficient for cluster formation. While the role of CheW in cluster formation has not been explored, the complete lack of swimming and chemotaxis in a *V. parahaemolyticus* Δ*cheW* mutant suggests that this protein is important for cluster formation and cannot be replaced by ParP in the signal transduction to CheA (Ringgaard *et al.* 2014). The absence of ParP, or ParC, leads to a reduction in swimming efficiency further suggesting that the ParP function is ultimately to enhance chemotaxis by ensuring the inheritance of a chemosensory cluster by each daughter cell.

The ParC-mediated localization of *Vibrio* chemosensory proteins is an active mechanism, as it requires ATP hydrolysis by ParC (Ringgaard *et al.* 2011). 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 ParAI and FlhG enabling their targeting at the cell pole (Yamaichi *et al.* 2012). Beside facilitating its partitioning upon cell division, it has been proposed that the biological significance of targeting CSS at the cell poles in *Vibrio* is to associate it spatially with the polar flagellum (Yamaichi *et al.* 2012). 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 (Lipkow, Andrews and Bray 2005), but the possible effect of positioning a chemosensory cluster at the opposite pole to the flagellum on response kinetics has not been tested.

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

As previously mentioned *R. sphaeroides* has two sets of chemosensory proteins organized into spatially separate arrays: one transmembrane and polar, the other soluble and cytoplasmic (Wadhams *et al.* 2003). CheAs and CheWs are required for the transmembrane cluster, whereas only CheW is required for the cytoplasmic cluster (Wadhams *et al.* 2005). At the beginning of the cell cycle cells possess a single cytoplasmic cluster, which is broadly centered about the mid-cell (Fig. 7D and E). The positioning of this cytoplasmic cluster is ensured by the TlpT cytoplasmic chemoreceptor, CheA2, CheW3 and CheW4 (Wadhams *et al.* 2002; Jones and Armitage 2017). Longer cells possess more TlpT clusters with the cluster number increasing proportionally with the cell length (Jones and Armitage 2017). This evidence indicates spatial limitations on the number of arrays in a cell and stochastic assembly similar to *E. coli*.

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) (Fig. 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 chemosensory proteins (Fig. 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 coding 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).

Beside being essential for the segregation of the cytoplasmic cluster, PpfA might also have a role in facilitating TlpT localization (Jones and Armitage 2017).

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 chemosensory 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 chemosensory 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 (Chiu *et al.* 2013). Sphaeroplasts have evenly spaced clusters again suggesting a nucleation and reaction-diffusion mechanism, as seen in *E.* coli (Chiu *et al.* 2013). 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 (Tucker *et al.* 2010). Such shape would generate a small region of high curvature that could accommodate the transmembrane receptor trimers of dimers.

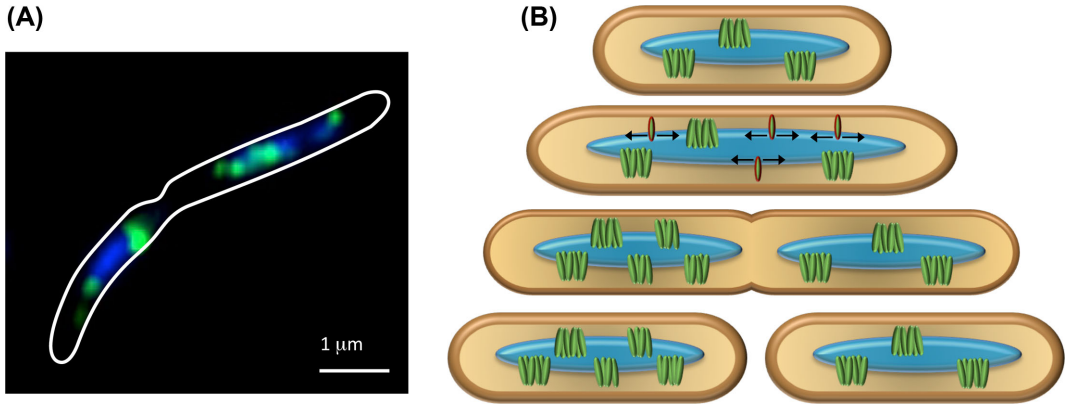

Figure 8. (**A**) *M. xanthus* FrzCD-GFP localization at the nucleoid. Fluorescence micrographs of a *M. xanthus* diving cell carrying a FrzCD-GFP fusion (green) stained with the DNA-DAPI staining (blue). The white contours have been manually drawn based on the corresponding bright-field micrograph (not shown). (**B**) A model showing the stochastic assembly of Frz chemosensory clusters (green) on the nucleoid (blue) and their segregation upon cell division.

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, 2017). Interestingly, FrzCD clusters do not occupy the whole cell length but only the central region where they colocalize with the nucleoid (Fig. 8A). Once associated with the nucleoid, FrzCD recruits FrzE (CheA) (Moine *et al.* 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 (Moine *et al.* 2017). Like other bacteria, cluster formation also requires the presence of the CheA-like FrzE (Moine *et al.* 2017).

The Frz cluster formation on the nucleoid might occur in a stochastic manner similarly to the assembly of the *E. coli* chemosensory arrays in the membrane. Indeed, the binding of FrzCD to DNA can 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 *et al.* 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 by mechanisms similar to those of transmembrane chemoreceptors using the bacterial inner membrane as a platform to form the arrays of trimers of dimers (Briegel *et al.* 2012). It has been recently suggested that the CheW-like FrzB might serve as a stabilizing factor of Frz nucleoid clusters as in the absence of this protein Frz foci results less defined (A divergent CheW stabilizes nucleoid-associated chemosensory arrays — bioRxiv). Indeed, for the correct activation and stabilization of chemosensory arrays at the nucleoid an extra stabilizing factor might be required. Such stabilizing factor would be dispensable when receptors arrays are tightly embedded in the inner membrane. A case analogous to the one proposed is represented by the *V. cholerae* cytoplasmic chemotaxis cluster 1, whose stabilization is guaranteed by the presence of an additional signaling domain in its DosM receptor (Briegel *et al.* 2016).

One outcome of the nucleoid-driven Frz 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 signal amplification. In fact, increasing concentrations of an Frz effector induce a dose-dependent response that suggests the presence of signal amplification. In cells lacking the nucleoid binding domain, the reversal frequencies increase linearly with the effector doses, suggesting that, in this case, signaling is only function of the number of activated receptor-signaling complexes dispersed in the cytoplasm (Moine *et al.* 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).

POSITIONING OF CSS AND 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 *Pseudomonas aeruginosa*, possess multiple chemosensory clusters (Güvener and Harwood 2007; O'Connor *et al.* 2012; Moine *et al.* 2014). 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*) (Burrows 2012; Schumacher and Søgaard-Andersen 2017) and FAC (for *M. xanthus*) (Faure *et al.* 2016) 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 support to this hypothesis, while short swimmer cells of the species *Vibrio parahemolyticus* carries a single polar chemosensory array and a single polar flagellum, longer swarmer cells from the same species develop multiple distributed later clusters and peritrichous flagella (Heering and Ringgaard 2016). Interestingly and unlike *E. coli*, Vibrio lateral clusters do not form simply as a consequence of the elongated state of swarmer cells, but seem to be produced as a part of a specific differentiation program (Heering and Ringgaard 2016).

In *R. sphaeroides*, the presence of two chemosensory clusters with different chemoreceptor 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. 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 chemosensory cluster to recruit the correct set of proteins (Scott *et al.* 2010; Moine *et al.* 2014).

CONCLUDING REMARKS

While the primary function of CSS clusters is to reach a maximal sensitivity to signals and to produce a cellular response that is amplified as compared as the amount of initial signal, their subcellular localization has been probably selected to ensure the inheritance of transmembrane and cytoplasmic clusters within the bacterial population. For transmembrane polar arrays, while the determinants of cluster positioning varies among species, in all cases cluster inheritance will occur along with that of the cell poles (Thiem, Kentner and Sourjik 2007; Thiem and Sourjik 2008; Ringgaard *et al.* 2011; Yamaichi *et al.* 2012; Santos *et al.* 2014; Strahl *et al.* 2015; Neeli-Venkata *et al.* 2016; Alvarado *et al.* 2017; Draper and Liphardt 2017; Saaki, Strahl and Hamoen 2018).

In the case of cytoplasmic clusters, their inheritance is ensured by active segregation mechanisms like those described for plasmid partitioning (Thompson, Wadhams and Armitage 2006; Roberts *et al.* 2012). Alternatively, the presence of multiple distributed clusters on the nucleoid allows to bypass the need for a faithful segregation mechanism with clusters being inherited passively and along with the nucleoids (Moine *et al.* 2017).

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