Chapter 5

The phospho-regulated scaffolding protein CheV affects chemoreceptor clustering in *Salmonella typhimurium*

The transmembrane receptors in the bacterial chemotaxis circuit interact with the central kinase CheA via scaffolding proteins CheW and CheV. CheV is a hybrid protein that contains a phosphorylatable receiver domain and plays a role in receptor clustering and adaptation to chemoeffectors in some bacterial species. Here we study the function of CheV in the chemotaxis of *Salmonella typhimurium*. In the absence of methylation-dependent adaptation, cheV knockout cells spread slower, and the CheV+ phenotype is restored only by expression of CheV that can be phosphorylated. In vivo fluorescence resonance energy transfer measurements indicate a weak but significant CheV-dependent partial adaptation to the non-metabolizable attractant α-methyl-aspartate. The adaptational recovery, however, is not affected by CheV phosphorylation. Using fluorescently-tagged CheV, we show that CheV forms clusters, predominantly localized at the cell poles, and the number and localization of these clusters does not depend on the phosphorylation state. Using YFP-CheR as a marker for receptor clusters, however, we show that the receptor cluster number decreases in cheV knockout cells, and has intermediate levels in a phosphorylation-deficient CheV mutant strain. The number of lateral receptor clusters also decreases in the absence of CheV. We speculate that CheV might play a role in the clustering of some of the chemoreceptor species in *S. typhimurium* and in the adaptation to chemoeffectors which these chemoreceptors can sense.
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5.1. Introduction

Scaffolding proteins bring together and promote interactions between different components of intracellular signaling networks. Signaling scaffolds create microenvironments in which the concentration of the participants is enriched, promote economical use of a limited set of signaling molecules without compromising specificity, and can also play active role in network-level dynamic regulation of the signaling modules involving feedbacks. Scaffolding proteins are ubiquitous in eukaryotic signaling, but also play role in some signaling processes in bacteria: for example CheW and CheV are proteins that couple the transmembrane chemoreceptor arrays to a cytoplasmic layer of the complex formed by the histidine kinase CheA.

In the two-component chemotactic signaling system of bacteria, chemoeffectors are detected by transmembrane chemoreceptors that form allosteric complexes with histidine kinase molecules (CheA). Activated CheA gets autophosphorylated, and transfers phosphoryl groups to the response regulator CheY. Phosphorylated CheY in turn modulates the frequency of clockwise rotation of the flagellar motors. Different enzymes that accelerate CheY dephosphorylation, and enzymes that promote adaptation to chemoeffectors by reversible covalent modification (methylation) of chemoreceptors at multiple sites, exist in different bacterial species. In all organisms that have chemotaxis networks defined by the presence of CheA, the kinase is coupled to the chemoreceptors by the scaffolding protein CheW, CheV or both, and these proteins are essential for the receptor-kinase interactions.

CheW interactions with CheA and the chemoreceptors have been extensively studied: CheW binds in vitro to both with dissociation constants ~6 µM and ~11 µM respectively, forming stable ternary complexes. Chemoreceptors of different types form mixed trimers of dimers, which couple to CheA via CheW to form higher-order signaling arrays. High levels of expression of CheW beyond the functional chemoreceptor-CheW-CheA complex stoichiometry, impair chemotactic ability of bacteria. A recent study proposes that CheW-binding sites in receptor dimers overlap their trimer contact sites and overexpression of CheW saturates the
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inter-receptor-binding sites, preventing the assembly of trimers-of-dimers units. CheW plays a role also in the formation of higher-order receptor assemblies, which detailed structure has been recently revealed by cryoelectron tomography.

CheV is a two-domain protein (Figure 5.1A), in which the N-terminal domain is homologous to CheW (hereafter referred to as Vw). CheW residues that are crucial for the coupling with CheA and chemoreceptors have been identified, and multiple alignment shows that several of these residues are conserved between CheW and Vw. This observation suggests that CheV could also interact with CheA and the chemoreceptors, although such interactions have not been proven experimentally. The C-terminal domain of CheV (hereafter referred to as REC) is homologous to regulatory proteins that are phosphorylated at a conserved aspartate residue, allowing them to switch between active and inactive conformations. The REC domains of CheV in some species, such as *Bacillus subtilis* and *Helicobacter pylori*, are phosphorylated in vitro.

The phenotypes of cheV knockout mutants differ between organisms, suggesting that the function and importance of CheV in different species varies. As a hybrid protein, CheV possibly performs a coupling function associated with its Vw domain, a regulatory/adaptation-related function associated with its REC domain, or another function that individual domains alone cannot support. For example, in *B. subtilis*, CheV phosphorylation is required for normal adaptation to asparagine. CheV also has a receptor-coupling function in *B. subtilis*, however, its function is partially redundant with CheW: both ΔcheV and ΔcheW knockouts but not ΔcheV ΔcheW double knockout are chemotactic.

In *H. pylori*, which has three CheV proteins, loss of CheW leads to a non-chemotactic phenotype, whereas the loss of each of the three CheVs does not impair chemotaxis abilities of the cells, although their motility phenotypes are different. *H. pylori* lacks a methylation-dependent adaptation system, and it is possible that some of its CheV proteins are involved in a methylation-independent adaptation mechanism.

Here we study the role of CheV in chemotaxis of *S. typhimurium*. CheV has been recently identified in *S. typhimurium* and deletion of
cheV in wild type cells produces only very subtle phenotypic changes. S. typhimurium cells in which the genes of the enzymes CheR and CheB involved in methylation-dependent adaptation were knocked out, form chemotactic rings in soft-agar motility assays, suggesting that a methylation-independent mechanism of adaptation might exist. The later discovery of CheV suggested that this protein might be involved in an adaptation that works in parallel to the methylation-dependent pathway in wild type cells. CheV is the only cytosolic component of the chemotactic network of S. typhimurium that does not have a homolog in the orthologous chemotaxis system of its close relative Escherichia coli (Figure 5.1B). However, S. typhimurium has more chemoreceptor species than E. coli (see Table 3.1, Chapter 3), so it is possible that CheV is involved only in the coupling of these chemoreceptors species not present in E. coli to the kinase CheA.

We investigated the phenotype of CheV mutant strains in soft-agar plates and showed that CheV, but not a phosphorylation-deficient CheV mutant can restore the phenotype of an adaptation-deficient ΔcheR ΔcheB ΔcheV strain to that of ΔcheR ΔcheB strain. Our measurements of the kinase activity indicate a weak but significant partial adaptation upon stimulation with α-methyl-aspartate (MeAsp) steps in methylation-deficient cells that express CheV. Imaging reveals that fluorescently labelled CheV and its non-phosphorylatable variant CheVD250A both form clusters in the presence of chemoreceptors. Moreover, knocking out cheV affects receptor-cluster number and localization. We discuss possible roles of CheV in chemoreceptor clustering and adaptation to chemoeffectors sensed by different chemoreceptor species.
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Figure 5.1. Structure of CheV and topology of *S. typhimurium* chemotactic network. (A) Domain structure of *S. typhimurium* CheV. Domain boundaries were determined using the SMART database. \(^{141,217}\) The N-terminal domain (the amino acids 1-186), Vw, is homologous to CheW. The amino acids 187-313 form a receiver (REC) domain, which might be phosphorylated at D250. The phosphorylatable aspartate position was determined by aligning of CheV and CheY: the site of phosphorylation D57 of CheY corresponds to D250 in CheV (all the conserved residues among the CheY family of response regulators are either identical in CheV or are conservative substitutions). Multiple alignment of CheV proteins from different species show that the D250 site is conserved (M.Sc. thesis, M.E. Dougherty, 2006). (B) Chemotaxis network of *S. typhimurium*. Network topology is identical to that of *E. coli*, except for the presence of CheV (V), which could be phosphorylated by the kinase CheA (A), and could shuttle in and out of the receptor-kinase complex. The other proteins in the chemotactic network are the chemoreceptors (methyl-accepting chemotaxis proteins, MCPs), the response regulator CheY (Y) and its phosphatase CheZ (Z), the scaffolding protein CheW (W), the methyltransferase CheR (R) and methylesterase / deamidase CheB (B).
5.2. CheV enhances the spreading of methylation-deficient cells in soft-agar assays

To identify the role of CheV in chemotaxis of *S. typhimurium*, we performed a soft-agar assay, in which the bacteria are inoculated in tryptone broth (TB) low-concentration agar and subsequently spread outwards due to growth and chemotaxis in self-created gradients, formed by metabolizing the surrounding nutrients. The phenotype of the ΔcheV knockout in a wild type background does not differ from that of wild type (Figure 5.2A). However, in knockout strains of the adaptation enzymes CheR and CheB, we noticed a phenotypic difference: whereas ΔcheR ΔcheB strains spreads outwards, forming a single sharp ring, the ΔcheV ΔcheR ΔcheB spreads much slower, forming a smaller single ring which is more diffuse than that of the ΔcheR ΔcheB strain (Figure 5.2B). A similar observation was reported earlier also by Wang et al. The phenotype of the ΔcheR ΔcheB strain is restored in ΔcheV ΔcheR ΔcheB expressing CheV from a plasmid (Figure 5.2C). Overexpressing CheV from a plasmid in ΔcheR ΔcheB does not change its phenotype (Figure 5.2C). CheV, fused with yellow fluorescence protein, CheV-YFP, also complements ΔcheV ΔcheR ΔcheB (Figure 5.2C), similar to the untagged CheV. However, a version of the same fusion protein that bears a point mutation in the putative CheV phosphorylation site (D250), CheV D250A-YFP, does not complement the ΔcheV phenotype in the ΔcheR ΔcheB background (Figure 5.2C), suggesting that phosphorylation is essential for the function of CheV in modulating chemotactic behavior.

CheV expressed in wild type *E. coli* does not affect substantially its spreading phenotype: if anything, both wild type CheV and the non-phosphorylatable version, CheV D250A, slightly increase the diameter of the outer spreading ring (Figure 5.2D). Both CheV and CheV D250A fail to restore the chemotactic abilities of cheW knockout of *E. coli*, which does not form chemotactic rings in TB soft-agar plates. In contrast to *S. typhimurium*, *E. coli* strains that do not express the methylation and demethylation enzymes CheR and CheB, do not form chemotactic rings on TB soft-agar plates. We expressed CheV and CheV D250A from a plasmid in ΔcheR ΔcheB *E. coli*, and only the strain expressing wild type CheV showed a minor enhancement in
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spreading after three days of incubation. The aforementioned experiments with *S. typhimurium* and *E. coli* suggest that CheV plays a role in chemotaxis, and its function depends on its abilities to be phosphorylated.

**Figure 5.2. Soft-agar assays of chemotactic behavior.** (A) Wild type and ΔcheV (ΔV) *S. typhimurium* after 7 h incubation. (B) ΔcheR ΔcheB (ΔRB) and ΔcheV ΔcheR ΔcheB (ΔV ΔRB) *S. typhimurium* after 9 h of incubation. (C) Complementation of ΔV ΔRB *S. typhimurium* (upper left) with CheV (V) expressed from a plasmid (upper middle) after 9 h of incubation. Upper right: CheV (V) from a plasmid was expressed in the ΔRB *S. typhimurium* and also imaged after 9 h of incubation. Lower: Complementation of ΔV ΔRB *S. typhimurium* with CheV-YFP (V-YFP) and CheV_D250A-YFP (V_P-YFP) after 9 h (left) and 19h (right) of incubation. ΔRB and ΔV ΔRB were also inoculated for comparison (D) Left: Comparison of the spreading of wild type *E. coli* (Ec) and *E. coli* in which CheV (V) and CheV_D250A (Vr) were expressed, after 7 h of incubation. ΔcheW *E. coli* (Ec ΔW) does not spread, and it is not complemented by either V or Vr. Right: ΔcheR ΔcheB *E. coli* (Ec ΔRB) expressing V shows a minor enhancement of its spreading in soft-agar compared to Ec ΔRB after >72 hours of incubation (due to the prolonged incubation and high density of bacteria it is hard to determine whether the spreading is diffuse or a chemotactic ring exists).
5.3. Role of CheV in adaptation to MeAsp

The formation of chemotactic rings in ΔcheR ΔcheB S. typhimurium strains has been interpreted as a methylation-independent adaptation pathway \(^{250,249}\). Consistent with this view, experiments with tethered ΔcheR ΔcheB cells showed partial recovery of the motor rotational bias upon stimulation with a chemoeffectector step \(^{250,249}\). CheV had not yet been discovered at the time of those studies, but later investigation of the role of CheV in methylation-deficient cells \(^{267}\) showed that ΔcheR ΔcheB S. typhimurium has a near-normal tumbling bias, whereas ΔcheV ΔcheR ΔcheB has a higher counterclockwise bias of its motors, i.e. they change direction (tumble) less frequently. Tumblier cells migrate faster on soft-agar plates in chemotaxis-deficient strains \(^{276}\), which can provide an explanation of the different migration phenotype of the two strains. Another explanation for the partial adaptation of ΔcheR ΔcheB cells could be the adaptive remodelling of the flagellar motor, recently discovered by Yuan et al \(^{289}\).

We have shown that the time scales of adaptation to MeAsp in the wild type and cheV knockout strain do not differ significantly: the frequency response of the two strains are nearly identical (see Chapter 3, the characteristic frequency of the frequency response is determined by the adaptation time scale of the chemotaxis system \(^{260}\)). To test for adaptation of the kinase activity in the absence of receptor methylation, we applied steps of chemoeffectectors to ΔcheR ΔcheB S. typhimurium strains and observed the changes in the kinase (CheA) activity using in vivo fluorescence resonance energy transfer (FRET). The assay utilizes a FRET pair between the phosphatase CheZ and the response regulator CheY, fused to yellow and cyan fluorescent proteins (YFP and CFP) respectively, and provides a real-time readout of the kinase activity \(^{242}\). We tested the response to a step stimulus of the non-metabolizable attractant MeAsp, and we observed a minor but reproducible adaptational recovery only in the presence of CheV. Figure 5.3A shows a typical FRET response time series of the ΔcheR ΔcheB strain expressing wild type CheV, CheV\(_{D250A}\) or no CheV to 0.25 mM MeAsp. Both the strain expressing wild type and mutant CheV show partial adaptation recovery of \(~10\%\). ΔcheV strain does not show partial
adaptation even after >1500 s (Figure 5.3A), suggesting that CheV could be involved in a methylation-independent adaptation mechanism. The degree of partial adaptation in CheV-containing strains is minor and only slightly greater than the measurement noise, and occurs on a very slow time scale (>1500 s), whereas the tethering assays of Stock et al. demonstrated adaptation on a ~100 s time scale.

The smaller amplitude of the response in the ∆cheV strain to 0.25 mM MeAsp suggests that the sensitivity of this strain to MeAsp could be lower. We constructed dose-response curves for ∆cheR ∆cheB strains with and without the cheV gene. We measured the initial amplitude of the FRET response (FRETᵢ), normalized it to the saturated response to a mix of MeAsp and serine (|FRETᵢ|max), inhibiting the two major chemoreceptors Tar and Tsr, and plotted FRETᵢ/|FRETᵢ|max as a function of the MeAsp concentration (Figure 5.3B, negative responses; the |FRETᵢ|max is -0.11±0.05 and -0.14±0.07 for the strains with and without CheV respectively). Although the shapes of the tested curves for the two strains are similar, the dose-response curve of the cheV knockout is shifted towards the higher concentration range, indicating lower sensitivity to MeAsp. For both strains, two dissociation constants (Kᵦₛ) are apparent, and the dose-response curves are fit by a multisite Hill model

$$y = y'^I \frac{x^n_I}{K^I_x^n_I + x^n_I} + y'^II \frac{x^n_{II}}{K^{II}_x^n_{II} + x^n_{II}}$$

where x is the concentration of MeAsp, y is the normalized FRETᵢ, y'I and y''I are the two plateaus in the normalized FRETᵢ level, K_I and K''_I are the two apparent dissociation constants, and n_I and n''_I are the two Hill coefficients. Hill coefficients of 1.1 and 1, and 1.2 and 1, are used for the fits shown on Figure 5.3B (for the plateaus reached at -0.8 and -1 and -0.7 and -1 respectively). The dissociation constants for ∆cheR ∆cheB strain are 0.17 and 400 mM, and for the ∆cheV ∆cheR ∆cheB strain: 0.40 and 409 mM MeAsp. We have shown that in S. typhimurium more than one receptor mediates responses to MeAsp (see Chapter 3). Since the receptors in both tested strains are in the same modification state due to lack of CheR and CheB, the difference in the sensitivity could be explained by a different proportion of receptors that are coupled in receptor-scaffold-kinase complexes, for example if CheV is involved in coupling of one of the receptors involved in MeAsp sensing but not the other(s).
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Figure 5.3. FRET response of *S. typhimurium* to chemoeffector steps. (A) Representative time series of the FRET response of ∆cheR ∆cheB (∆RB), ∆cheV ∆cheR ∆cheB (∆V ∆RB) and cheV_D250A ∆cheR ∆cheB (VP ∆RB) strains to 0.25 mM MeAsp. MeAsp is added and removed at times 0 on the left and right part of the panel, respectively. (B) Normalized initial amplitude of the FRET response (FRET\(_0\)) as a function of MeAsp concentration for ∆RB and ∆V ∆RB strains. Fits to a multisite Hill equation of the MeAsp response of ∆RB and ∆V ∆RB are shown (see text for fitted parameters).

5.4. Phosphorylation-independent localization of CheV at the cell poles

In order to explore the role of CheV in coupling and adaptation at the molecular level, we studied the localization of CheV and its non-phosphorylatable mutant CheV_D250A, using fluorescent fusion proteins CheV-YFP and CheV_D250A-YFP, expressed in ∆cheV ∆cheR ∆cheB *S. typhimurium*. The functionality of the CheV-YFP fusions were confirmed by the complementation of cheV deletion in soft-agar assays (Figure 5.2C). We observe that both CheV and CheV_D250A form cluster-like aggregates, but these clusters are not observed in cells in which most of the chemoreceptors are knocked out (Figure 5.4A; ∆7 refers to *S. typhimurium* strain, which has only McpA and Tip chemoreceptors). The existence of CheV-YFP clusters only in cells that contain chemoreceptors suggest that these clusters can serve as markers of chemoreceptor-scaffold-kinase complexes.
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**Figure 5.4.** Localization and clustering of CheV-YFP and CheVD250A-YFP in *S. typhimurium*. (A) Representative fluorescence microscopy images (600x) of CheV-YFP (left) and CheVD250A-YFP (middle) in ΔcheV ΔcheR ΔcheB ΔV ΔRB strain. Right: Representative image of CheV-YFP in *S. typhimurium* cells, in which seven of the nine chemoreceptors are knocked out. (B) Upper row: distribution of the number of clusters formed by CheV-YFP and CheVD250A-YFP in ΔV ΔRB cells. 137 and 141 cells were analysed respectively. Lower left: mean number of receptor clusters per cell. Lower right: percent of cells with > 2 clusters. (C) Localization of clusters of CheV-YFP and CheVD250A-YFP in ΔV ΔRB cells. White, blue and red bars represent the percent of cells with no clusters, no lateral clusters and at least one lateral cluster (see text). The error bars in (B) and (C) represent the standard deviation obtained from two independent repeats (77 and 60 cells expressing CheV-YFP, and 69 and 72 cells expressing CheVD250A-YFP).
We evaluated the number and localization of the detectable CheV-YFP and CheV\textsubscript{D250A}-YFP clusters within individual cells. 137 and 141 cells respectively were analyzed for each fusion protein in \textDelta{cheV} \textDelta{cheR} \textDelta{cheB} \textit{S. typhimurium}. For both constructs, we determined the distribution of cluster numbers. The mean number of detected clusters per cell is \~1.2 and \~8\% of the cells contain >2 clusters (Figure 5.4B). Differences, if any, are within the experimental error. To determine the position of the clusters within each cell, we divided the cells in three parts along the long axis, and considered clusters within the inner third lateral, and within the two outer thirds – polar. The distributions of the clusters among these partitions along the long cell axis were indistinguishable for the phosphorylatable and non-phosphorylatable CheV: cells containing at least one lateral cluster make up \~30\% of the total population (Figure 5.4C). We conclude that the localization and number of the CheV-containing clusters does not depend on phosphorylation of the protein. However, we cannot rule out subtle differences in the number of receptors per cluster, which could be measured in future studies for example using superresolution microscopy \textsuperscript{94} to count the number of CheV molecules per cluster.

5.5. The number of receptor clusters is smaller in \textit{cheV} mutant cells

To obtain single-cell statistics of the number and localization of receptor clusters in the presence and absence of CheV, we used a YFP fusion of the methyltransferase CheR as a reporter \textsuperscript{119}. CheR binds to the C-terminal conserved pentapeptide found at four of the chemoreceptors in \textit{S. typhimurium} (Tar, Tsr, Tcp, and McpB, see Chapter 3, Table 3.1). The YFP-CheR fusion does not appear to interfere with clustering \textsuperscript{229}, therefore it can be used to observe the localization of both clustered and non-clustered chemoreceptors \textsuperscript{119}.

We imaged YFP-CheR in wild type, \textDelta{cheV} knockout and \textit{cheV\textsubscript{D250A}} non-phosphorylatable mutant \textit{S. typhimurium} strains (Figure 5.5A). Fluorescence clusters are observed in all three strains; however, there are differences in the number of clusters per cell and in the cluster localization (Figure 5.5B, C). We obtained the distribution of cluster number per cell (for 507, 452, and 676 cells per strain respectively). The mean number of
CheV affects chemoreceptor clustering in *Salmonella typhimurium* clusters is the highest in wild type cells (1.4±0.1), intermediate in cells, expressing non-phosphorylatable CheV_{D250A} mutant (1.2±0.1), and the lowest in ΔcheV knockout cells (0.9±0.1) (Figure 5.5B; the error bars represent the standard deviation obtained from two independent repeats). The number of cells with more than two clusters is also decreasing in order of wild type, cheV_{D250A} strain and ΔcheV knockout strain: 14±6, 8±5, and 4±0.2 percent of the total number of cells, respectively (Figure 5.5B; the error bars represent the standard deviation obtained from two independent repeats). The decrease in cluster number reflects the smaller number of lateral clusters observed in cheV mutant strains: 30±1% of the wild type cells contain at least one lateral cluster, whereas the fractions of cells containing at least one lateral cluster is 21±3% in cells that express the non-phosphorylatable CheV_{D250A} mutant, and 15±3% in the ΔcheV knockout cells (Figure 5.5C). We concluded that CheV increase the chemoreceptor clustering in a phosphorylation-dependent manner, and the role of CheV in chemoreceptor clustering is particularly pronounced for the clusters with a lateral localization.
Figure 5.5. Localization and clustering of YFP-CheR. (A) Representative fluorescence microscopy images (150x) of YFP-CheR in wild type (WT, left), ΔcheV (ΔV, middle) and cheV250A (Vp, right) S. typhimurium strains. (B) Distribution of the number of YFP-CheR clusters in WT (upper left), ΔV (upper middle), and Vp (upper right) cells. 507, 452, and 676 cells were analysed respectively. Lower left: mean number of receptor clusters per cell. Lower right: percent of cells with more than two clusters. (C) Localization of YFP-CheR clusters in WT, ΔV, and Vp cells. The error bars in (B) and (C) represent the standard deviation obtained from two independent repeats (132 and 375, 87 and 365, and 123 and 553 cells for WT, ΔV, and Vp respectively).
5.6. Discussion

We have explored the role of the scaffolding protein CheV in adaptation and chemoreceptor clustering in *S. typhimurium*. Using classical chemotaxis soft-agar assay, we have demonstrated a phenotypic difference between CheV+ and CheV- cells, incapable of methylation-dependent adaptation (Figure 5.2). The difference is complemented by CheV but not by its non-phosphorylatable mutant CheV\textsubscript{D250A}, suggesting a role of phosphorylation in the functional role of CheV. Faster spreading in the presence of CheV could be explained either by partial adaptation dependent on CheV (Figure 5.2A), and/or by the higher counter-clockwise bias of the flagellar motors of CheV- cells, which was reported previously. CheV expression promotes the formation of chemotactic rings in soft-agar experiments even in methylation-deficient *E. coli* cells, which is not achieved by the mutant CheV\textsubscript{D250A}. This finding suggests that CheV might be involved in the clustering or functioning of some of the receptors common to *E. coli* and *S. typhimurium*, *i.e.* Tar, Tsr, Trg or Aer.

We have studied the role of CheV in *S. typhimurium* chemotaxis signaling using *in vivo* FRET measurements in CheV+ and CheV- cells (Figure 5.3). In Chapter 3 we have probed the frequency response of the chemotaxis system in CheV+ and CheV- cells (Chapter 3, Figure 3.6), and have not observed differences in the characteristic frequency, which is inversely proportional to the adaptation time scale of the chemotaxis system. Here we probed the adaptational recovery to steps of α-methyl-aspartate, and observed a partial (~10%) adaptation in CheV+ cells, even when the phosphorylation site of CheV is disrupted. The adaptational recovery is small, comparable with the experimental noise; thus assays of chemotactic signaling with better sensitivity could be useful for precise quantification of the degree of adaptation. However, our experiments could provide an explanation for the Stock et al. studies reporting methylation-independent adaptation in *S. typhimurium*. The larger degree of adaptation observed in these studies, as well as the shorter adaptation time scale, on which the adaptation was observed by Stock et al., could be attributed to an additional adaptation mechanism that occurs downstream of the kinase activity. For example, a recent study by Berg’s
group\textsuperscript{289} reported methylation-independent adaptation at the level of the flagellar motor based on active remodelling of the composition of the flagellar motors. CheV might be involved in a similar adaptation mechanism but at the level of receptor-kinase complexes.

Another difference between the kinase activity of CheV\textsuperscript{+} and CheV\textsuperscript{-} cells is the lower sensitivity to MeAsp of the latter (Figure 5.3B). The shapes of the dose-response curves are similar, with two plateaus that suggest more than one receptor binding MeAsp (see also Chapter 3, Figure 3.9, showing Tar-independent responses to MeAsp). We speculate that CheV might affect to different degrees the coupling and clustering of the different chemoreceptors sensing MeAsp, for example if some chemoreceptor species form complexes preferentially with CheW and others - with CheV (see Chapter 6).

In order to assess the localization of CheV in \textit{S. typhimurium} cells, we created a CheV-YFP fluorescent fusion protein, the functionality of which was confirmed using soft-agar assays. We demonstrated that both CheV-YFP and the non-phosphorylatable variant CheV\textsubscript{D250A}-YFP form clusters only in cells containing the majority of the chemoreceptors (Figure 5.4). Clusters were predominantly localized in the poles of the cells. We have not identified a difference either in the cluster number or in the cluster localization using wild type CheV or its non-phosphorylatable mutant CheV\textsubscript{D250A}. However, we cannot exclude subtle differences for example in the number of receptors per cluster, which could be quantified in future studies using superresolution microscopy.

We have also assessed the number and localization of receptor clusters in CheV\textsuperscript{+} and CheV\textsuperscript{-} cells, using YFP-CheR as a marker for receptor clusters (Figure 5.5). Receptor cluster number is lower in cells where \textit{cheV} is knocked out, and also the clusters in the \textit{cheV} knockout strain are localized predominantly in the cell poles, whereas CheV\textsuperscript{+} cells have also a large fraction of lateral clusters. Cells expressing non-phosphorylatable CheV\textsubscript{D250A}, have more clusters than CheV\textsuperscript{-} cells, but fewer clusters than wild type cells; the number of lateral clusters follows the same pattern. These observations indicate that CheV plays a role in receptor cluster formation in \textit{S. typhimurium}, and phosphorylation of CheV is likely to increase the number of CheV-coupled chemoreceptors. A differential
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distribution of phosphorylated and non-phosphorylated CheV has been recently reported in *B. subtilis* \(^{279}\); phosphorylated CheV is predominantly localized in the lateral clusters, which might also be the case in *S. typhimurium*.

CheV does not complement cheW knockouts either in *E. coli* (Figure 5.2D) or in *S. typhimurium* where the cheW deletion abrogates chemotaxis (Chapter 4, Table 4.1). Thus, CheW’s and CheV’s role in chemoreceptor coupling of *S. typhimurium* are not redundant. Future studies with CheV and CheW co-localization for example by labelling with fluorophores with different colors, could reveal whether CheW and CheV participate in the same or in separate clusters.

In summary, we have shown that *S. typhimurium* CheV affects the number and localization of chemoreceptor clusters in a phosphorylation-dependent manner. We speculate that such changes could be explained by dynamic remodelling of the clusters, in which CheV acts as a scaffolding protein. Adaptive remodelling of the receptor-kinase clusters could be a consequence of changes in the phosphorylation level of CheV. Since the phosphorylation of CheV is most likely performed by CheA, and CheA activity is modulated by binding of chemoeffectors to the transmembrane chemoreceptors, CheV might be involved in a phosphorylation-dependent feedback regulation of the stability of the receptor clusters. Such a novel mode of feedback might explain the partial adaptation observed in methylation-deficient *S. typhimurium* cells, and this phosphorylation-dependent regulation could be more pronounced under certain environmental conditions. One possibility is that CheV and CheW are expressed to different degrees under different conditions of growth, or in different strains. Another possibility is that CheV and CheW are involved in coupling of different subsets of chemoreceptors. Since the chemoreceptor species change their relative abundance under different growth conditions or phases of bacterial growth \(^{210, 284}\), the role of CheV and CheW may also change depending of the relative chemoreceptor species abundance. Future studies of CheV expression and localization under controlled environmental conditions could reveal more insights regarding the function of CheV in *S. typhimurium*.
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5.7. Materials and Methods

**Bacterial strains and plasmids**

All strains and plasmids used in this study are listed in Table 5.1. All *S. typhimurium* strains, except the strain, in which seven chemoreceptors are removed from the chromosome (SM162, referred to as Δ7) have LT2 background. The Δ7 strain is used in CheV clustering experiments and we verified that its parent strain, 14028, CheV clusters in a manner similar to the clustering in LT2.

In-frame deletion of genes in the strains designed for this work was achieved by allele-replacement procedure based on Datsenko and Wanner’s method. *S. typhimurium* LT2 resident plasmid pSLT contains a ccdA ccdB operon that interferes with the deletion strategy. Thus, pSLT was first displaced using Kit10 from *Salmonella* Genetics Stock Collection (SGSC): a plasmid pLL6, which is from the same compatibility group as pSLT, is transformed in the strain of interest, pSLT is cured, and pLL6 is subsequently removed using temperature selection. The initial knockout step involves an insertion of a cassette providing kanamycin resistance, and also containing the lethal gene ccdB under the control of L-rhamnose inducible promoter. The cassette is removed by positive selection on L-rhamnose-minimal plates. All allele replacements were verified by genetic sequencing.

The plasmid for CheV expression (pML13) was constructed by PCR amplification of genomic cheV using primers, containing SacI and XbaI restriction sites for ligation into the same sites on the expression vector pBAD33. The plasmid for CheV_{D250A} expression (pML14) was created in similar manner, but D250A mutation was introduced using overlap PCR. Induction of protein expression from pML13 and pML14 was achieved using 0.01% L-arabinose. The plasmid for CheV-YFP expression (pML16) was created by overlap PCR, introducing a GSGGGG linker, which contains BamHI site, between cheV and eyfp genes. The overlap product was ligated in the expression vector pTrc99A using SacI and XbaI sites. The plasmid for CheV_{D250A}-YFP expression (pML17) was created in similar manner, but cheV gene was replaced with cheV_{D250A}. Induction of protein
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expression from pML16 and pML17 was achieved using 2 μM isopropyl-β-D-1-thiogalactopyranoside (IPTG). All plasmid constructs were verified by genetic sequencing.

The YFP-CheR fusion that served as a marker for receptor clusters was expressed from a plasmid pVS102, using 0.01% L-arabinose.

The CheY-YFP and CheZ-CFP fusions for the FRET experiments were expressed from a plasmid pVS88, induced with 150 μM isopropyl β-D-1-thiogalactopyranoside (IPTG). The same amount of IPTG was used in the soft-agar assays, whenever ΔcheY ΔcheZ strains were used. In all strains used in FRET experiments, chromosomal *cheY* and *cheZ* were deleted in order to prevent a competitive interaction between labelled CheY-YFP and CheZ-CFP, and unlabelled CheY and CheZ.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Relevant genotype</th>
<th>Source</th>
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</thead>
<tbody>
<tr>
<td>LT2</td>
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<td><em>Salmonella</em> Genetic Stock Center (SGSC)</td>
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<td>TSS530</td>
<td>LT2 cheV D250A</td>
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</tr>
<tr>
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<td>LT2 ΔcheR ΔcheB</td>
<td>This work</td>
</tr>
<tr>
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<td>LT2 ΔcheV ΔcheR ΔcheB</td>
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<td>TSS507</td>
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<td>R.M. Harshey</td>
</tr>
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<td><em>Escherichia coli</em> wild type for chemotaxis</td>
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<td>E. coli RP437 ΔcheW ΔcheY ΔcheZ</td>
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<table>
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<th>Resistance</th>
<th>Induction</th>
<th>Source</th>
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<td>ampicillin</td>
<td>IPTG</td>
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<td>cheV D250A-eyfp</td>
<td>ampicillin</td>
<td>IPTG</td>
<td>This work</td>
</tr>
</tbody>
</table>

**Table 5.1.** Strains and plasmids used in this work.
Chapter 5

Soft-agar plate assays

Bacteria were grown overnight to saturation in tryptone broth (TB, 1% Bacto tryptone, 0.5% sodium chloride, pH 7.0). Soft-agar (motility) plates were prepared using 25 ml TB per plate with appropriate antibiotics and inducers (Table 5.1), and solidified with 0.26% agar. Plates were left to cool down for ~2 h and 5 µl of the tested cultures were inoculated in the plates. Plates were then incubated at 30°C (the durations of the incubations are specified in the legend of Figure 5.2), and imaged using a custom-made dark-field imaging system with a Nikon camera.

Fluorescence microscopy and image analysis

Bacteria were grown at 33.5°C to a mid-exponential phase (OD600 ~ 0.5) in TB, supplemented with appropriate antibiotics and inducers (Table 5.1). Bacteria were harvested by centrifugation, washed and resuspended twice in motility buffer (10 mM potassium phosphate buffer pH 7.0, 0.1 mM EDTA, 1 µM L-methionine, 10 mM lactic acid, pH 7.0), and stored at 4°C 1-5 h prior to the experiment.

Bacteria were immobilized on a poly-lysine coated coverslip. The bacterial density was controlled such as individual cells can be imaged and segmented by the image analysis program. The coverslip was attached to a tunnel slide, and subsequently imaging was performed on an inverted microscope (Nikon Eclipse), equipped with oil-immersion objective (100x). For all images, additional 1.5x magnification was used. For CheV imaging experiments, an extra magnifier (4x) was also used. For fluorescence imaging, cells were excited using 515 nm laser with 50 ms exposure time.

The cluster numbers and distributions were obtained using MATLAB. After background correction, cells were segmented using morphological operations from Imaging Toolbox (MATLAB). Clusters were defined based on their intensity (at least three standard deviations greater than the mean) and size. Clusters were separated to polar and lateral in the following manner. First, the longer diameter of each cell was determined. Clusters, which projection was within the distance between the poles and
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1/3 of the longer diameter were defined as polar. The rest of the clusters were defined as lateral.

**Fluorescence resonance energy transfer (FRET) experiments and data analysis**

Bacteria, expressing the FRET donor-acceptor pair CheZ-CFP / CheY-YFP, were prepared in the same way as for the fluorescence imaging experiments. *In vivo* FRET microscopy on live bacterial populations was performed as described previously. This FRET assay provides a real-time readout of the kinase (CheA) activity, because it measures the concentration of the complex between the phosphorylated CheY and the phosphatase CheZ, and in steady state the activity of the phosphatase and the kinase are equal.

Bacteria, were immobilized on a poly-L-lysine–coated microscope coverslip, attached to the top surface of a flow cell, and kept under constant flow of motility buffer, generated by a syringe pump (Harvard Apparatus, PHD2000). Chemoeffectors were added and removed by using a fluidic switch (Hamilton, valve HV 3-2) that could rapidly select between input flow channels with different concentrations of chemoeffectors. There is a nearly constant delay of ~25 s between the time in which the change of solutions is induced by switching the valve (indicated as time 0 in the figures) and the time, when the new solution reaches the bacteria at the flow cell.

FRET microscopy on bacterial populations was performed on an upright microscope (Nikon FN1), equipped with an oil immersion objective (Nikon CFI Plan Fluor, 40x/1.3). The bacteria in the flow cell were illuminated by a metal halide arc lamp with closed-loop feedback (EXFO X-Cite exacte) through an excitation bandpass filter (Semrock, FF01-438/24-25) and a dichroic mirror (Semrock, FF458-Di01). The epifluorescent emission was split by a second dichroic mirror (Semrock, FF509-FDi01) into donor (cyan, C) and acceptor (yellow, Y) channels. The signals from the C and Y channels, passed through emission bandpass filters Semrock FF01-483/32 and FF01-542/27 respectively, were collected by photon-counting photomultipliers (Hamamatsu H7422P-40). Signal intensities were recorded
through a data acquisition card (National Instruments) installed on a PC, running custom-written software.

The ratio \( R \) between the coverslip background-corrected \( Y \) and \( C \) fluorescence signal intensities: \( R = Y/C \), provided an indicator of FRET activity, robust to fluctuations in the light intensity. The change in FRET activity upon stimulation, \( \Delta FRET \), can be expressed as a function of the change in the ratio \( \Delta R \),

\[
\Delta FRET = \frac{R_{pre} + \Delta R - R_0}{R_{pre} + \Delta R + |\Delta Y/\Delta C|} - \frac{R_{pre} - R_0}{R_{pre} + |\Delta Y/\Delta C|},
\]

where \( R_{pre} \) is the pre-stimulus acceptor to donor ratio, \( \Delta R = R - R_{pre} \) is the ratio change, \( R_0 \) is the acceptor to donor ratio in absence of FRET, and \( |\Delta Y/\Delta C| \) is the constant absolute ratio between the changes in the acceptor and donor signals per FRET pair, \( |\Delta Y/\Delta C| \approx 0.6^{242} \). Under the measurement conditions \( R_{pre} + |\Delta Y/\Delta C| \gg \Delta R \); thus \( \Delta FRET \sim \Delta R \). \( \Delta FRET \) is thus expressed in arbitrary units of \( \Delta R \).

5.8. Acknowledgements

Dr Sophie Roth provided help with development of image analysis code. Ilja Serjak, Marco Konijnenburg and Marco Seynen developed the dark-field imaging system.