

CheV1 Plays an Important Role in Chemotaxis of *Vibrio Cholerae*

Geetha Hiremath, So-Ichiro Nishiyama, and Ikuro Kawagishi

Abstract—The complete genome sequence of *V. cholerae* has revealed the presence of four CheV's, namely putative CheV, CheV1, CheV2 and CheV3, along with the three chemotaxis-related signaling systems designated as System I, II and III. To determine the roles of the CheV proteins, whose genes are located outside of the *che* gene clusters, we examined their cellular localization and then their roles in chemotaxis. Among the four CheV's, CheV1 plays a major role in chemotaxis as observed with swarm assay and fluorescence microscopy, suggesting its involvement in System II.

Index Terms—Chemotaxis, fluorescence microscopy, fusion proteins, localization, signal transduction, swarm assay.

I. INTRODUCTION

Vibrio cholerae, the etiological agent of cholera disease, is a highly motile bacterium possessing three sets of proteins with similarity to conventional chemotaxis-signaling proteins (Che proteins) and a single polar flagellum. Central to the chemotactic signal transduction, best studied in *E. coli* [1]- [5], is a two-component regulatory system where phosphate is relayed from histidine kinase CheA to the response regulator CheY with the help of other Che proteins, resulting in motor switch from counterclockwise to clockwise direction and hence reorientation of the cell. The receptors, which are homodimeric transmembrane proteins, localize at a cell pole and form a huge hexagonal array with a trimer of dimmers as building unit. CheW and CheA, as well as other Che proteins, target to the receptor cluster [6], [7] to form an exquisite signaling supramolecular complex or signalosome. Receptor clustering is critical for signal amplification and adaptation. The complete genome sequence of *V. cholerae* revealed the presence of several chemotaxis-related gene homologs [8]. Most of the ORFs encoding the different Che homologs are clustered in three different regions distributed on both chromosomes (Fig. 1). Similarly, several other organisms, including *M. Xanthus* [9], *P. aeruginosa* [10], *R. sphaeroides* [11] and others have been reported to contain multiple chemotaxis operons. Among the three Che Systems

of *V. cholerae*, System II alone directly mediates chemotaxis as deletion of CheA2 and also CheY3 of System II impaired chemotaxis [12], [13].

The genome sequence also showed a similar set of proteins other than the core signal transduction proteins, namely CheV proteins, which are not present in *E. coli* but are reported in *B. subtilis* [14], [15], *H. pylori* [16], [17], *S. enterica* [18]. These CheV proteins are the fusion proteins consisting of a N-terminal CheW-like (adaptor-like) and a C-terminal CheY-like (response regulator-like) domains. In *V. cholerae*, there are four CheV proteins namely, putative CheV, CheV1, CheV2 and CheV3, the genes of which are not in any of the three *che* gene clusters but are scattered over the genome. The *cheV* genes of *H. pylori* differentially affect motility [19] and *cheV* gene in *B. subtilis* is necessary for full chemoreceptor-CheA coupling and also for adaptation. The role of these *cheV* genes is not known in *V. cholerae* as none of them is located in any of the *che* clusters nor there is any data to show their involvement in chemotaxis. The aim of this study is to identify the roles of these CheV's, for which we first tried to characterize and group them into different Che Systems.

II. EXPERIMENTAL PROCEDURES

A. Bacterial Strains

The *V. cholerae* O395N1, a classical strain (Δ ctxA) [20] wild-type for chemotaxis, was used for all experiments. The *E. coli* strain DH5 α was used for cloning.

B. Construction of Plasmids

The target genes were cloned as in-frame fusions to the green fluorescent protein (GFP) gene by using pTrc-HisB-based plasmids, pTrc-HisB-GFP (for N-terminal fusion) and pDS1050 (for C-terminal fusion) vectors. The PCR amplified products were digested with restriction enzymes BsrGI-XbaI and NheI-XhoI, the resulting fragments were cloned between their respective restriction sites of pTrc-His-B and pDS1050 vectors. Expression of the fusion proteins were verified by immunoblot using anti-GFP antibodies. All insertions were verified by restriction analysis and sequencing.

C. Swarm Assay for Chemotaxis

Swarm assays were performed with tryptone semisolid agar (1% Bacto-Tryptone [BD], 0.5% NaCl, 0.3% Bactoagar [BD]) supplemented with 50 μ g/ml ampicillin and 100 μ M IPTG. A single colony or 2 μ l of overnight cultures were spotted onto the agar plate and incubated at 30°C for appropriate time.

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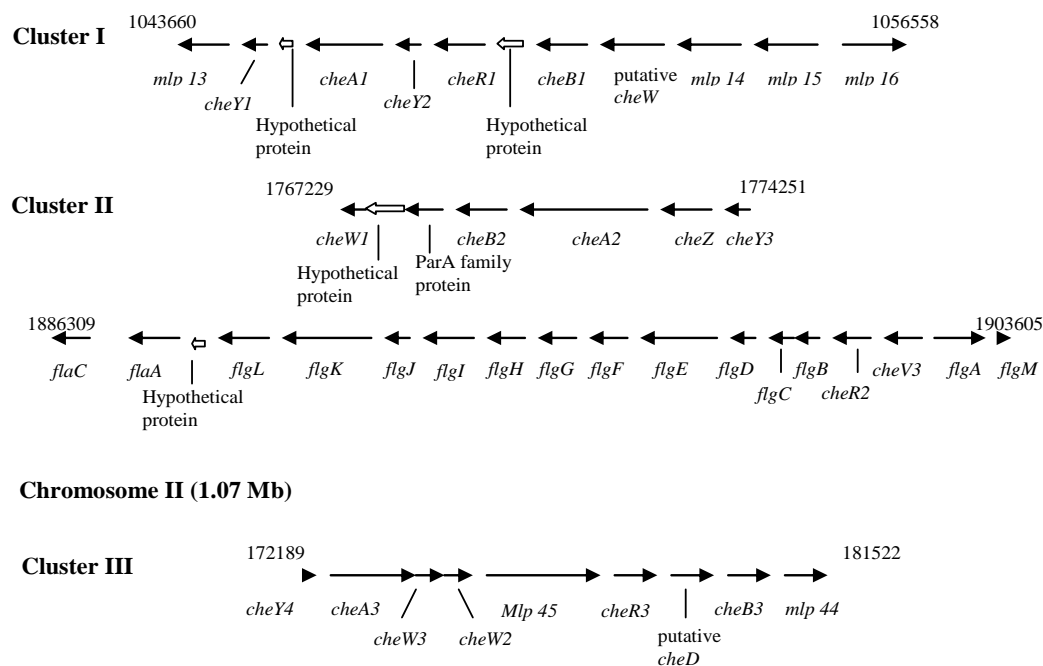
Chromosome I (2.9 Mb)

Fig. 1. A schematic diagram of the three che and fla gene clusters located on the two chromosomes in *V. cholerae* (classical biotype). Adapted from Gosink *et al.*, 2002

D. Growth Conditions and Fluorescence Microscopy

Cells were grown at 30°C in tryptone broth (1% Bacto Tryptone [BD], 0.5% NaCl, 0.5% [w/v] glycerol) supplemented with 50 µg/ml ampicillin. To express the proteins, overnight cultures were diluted 1:50 in TG medium with or without IPTG and were shaken for 4 hours. A cell suspension was applied to a coverslip and observed under a microscope (Olympus 1X71). The images were recorded and processed by using a cooled charge-coupled-device camera (Hamamatsu C10600) and the software Meta Morph version 7.6.

E. Immunoblotting

The samples were aliquoted from the fluorescence microscopy samples and checked for the protein expression. Proteins were denatured on SDS-PAGE and transferred onto PVDF membranes using semi-dry western blot transfer and detected using anti-GFP antibodies (Cell signaling).

III. RESULTS**A. Localization of System II and CheV Proteins**

The Initial analysis of the CheV proteins among the other bacterial species gave an idea about their involvement in chemotaxis. To understand their role in *V. cholerae*, GFP fusions to all the four CheV proteins were constructed and their subcellular localization was compared with the GFP fusions of Che components of Systems I, II and III using fluorescence microscopy. The fluorescence images showed that all the four CheV proteins localized to the pole, in a way similar to that of CheW1 of System II (Fig. 2A). All the proteins were expressed and detected by immunoblotting using anti-GFP antibodies (Fig. 2B). The CheW1 and other

System II components that are involved in chemotaxis constitutively localized to the pole, whereas the components of System I and III did not localize and were scattered throughout the cell (data not shown). These results suggest that CheV proteins might be involved in chemotaxis along with System II components.

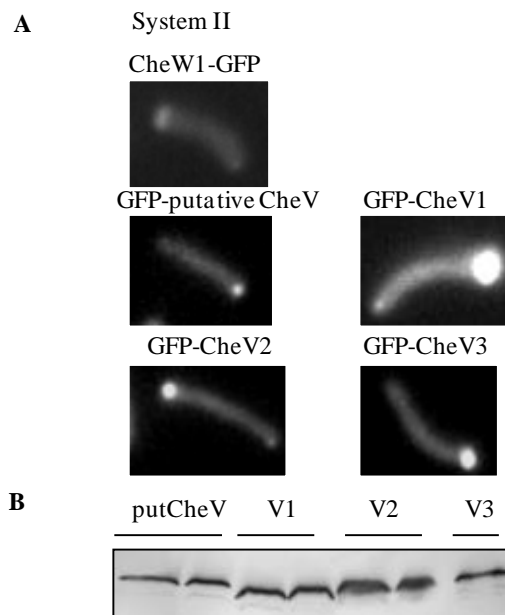


Fig. 2. Fluorescence images of CheVs and CheW1 fused to GFP in *V. cholerae*. Wild type (O395N1) cells expressing GFP-putative CheV (GFP-putCheV), GFP-CheV1, GFP-CheV2 and GFP-CheV3 were grown to late logarithmic phase and observed under microscopy (A). Immunoblotting of GFP fused CheV proteins. The constructed plasmids were introduced into wildtype(O395N1) strain. Lysates of cells were subjected to SDS-PAGE (15%) followed by immunoblotting with anti-GFP antibody (B).

B. Swarm Assay

To elucidate the roles of the four CheV homologs in *V.*

cholerae is difficult as none of their genes are located in any *che* cluster. Hence, we first tried to classify them into three different Che systems by checking the effects of their overexpression on swarming ability in a semisolid agar plate. The overproduction of System II components affects the chemotaxis of *V. cholerae* due to the jamming of the signaling system. To observe if CheV's behaved similarly, all the four *cheV* genes and the *cheW1* gene cloned downstream of the *trc* promoter were electroporated into the Che⁺ (O395N1) strain. A single colony from each transformant was stabbed into a 0.3% tryptone agar plate and observed for swarming. Whereas overproduction of CheV1 and CheV2 interfered with chemotaxis, putative CheV had a slight effect, and CheV3, which is located near the flagellar cluster, did not have any significant effect Fig.3. B1. Growth rates of cells overproducing each CheV homolog were similar to that of wild-type cells (data not shown). The average diameters of the swarm rings from three different experiments are plotted as shown in Fig. 3. B2 and are in the following order: pTrc >=

GFP-CheV3> GFP-putative CheV> GFP-CheV2> GFP-CheV1. CheV2 and CheV1 both interfered with swarming ability, where CheV1 showed a higher rate of interference indicating its role in chemotaxis.

III. CONCLUSIONS

The complete genome sequence of *V. cholerae* showed the presence of a unique set of modular proteins other than the core signal transduction proteins, namely CheV proteins, which are not present in *E. coli*, but are present in other bacteria such as *B. subtilis*, *H. pylori* and *S. typhimurium*. The presence of four CheV proteins along with the three Che systems indicates the presence of a complex signal transduction pathway in *V. cholerae*. In this study, we have focused on distinguishing CheV's into different systems and also to identify their role in chemotaxis. The presence of N-terminal CheW suggests its role in early stages of signal

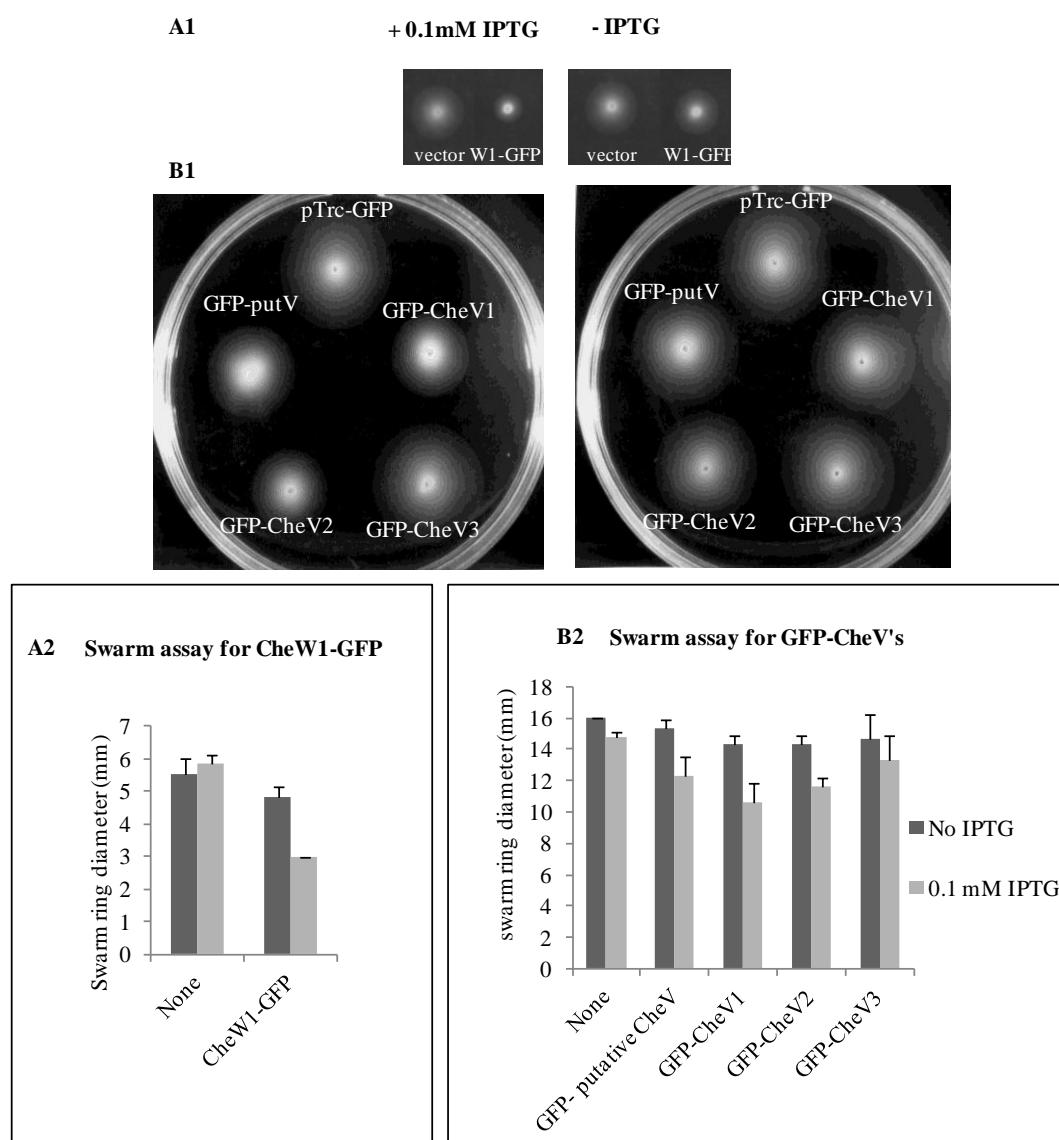


Fig. 3. Swarm assay. Swarming ability of overexpressed CheW and CheV fused to GFP proteins and the vector in wild type cells in the presence or absence of IPTG. A single colony from the transformants were stabbed onto ampicillin-supplemented 0.3% tryptone agar plates (in triplicates) without or with IPTG and incubated at 30°C (A1 and B1). The average diameter of the swarm rings from the three different experiments are plotted in the above graph with the error bars (A2 and B2).

Transduction. *V. cholerae* has four CheW proteins that are clustered into three different Systems, among which CheW1 of System II is involved in chemotaxis. In our study, we showed that CheV1 is an essential chemotaxis protein. Overexpression of CheV1 protein inhibited swarming ability of wild-type O395N1 cells as seen with the CheW1 protein. This is the same effect seen upon overexpression of the homologous *E. coli* CheW [21] where it has been clearly shown that overexpression of the protein is equivalent to its absence in swarming assay. The sequence alignment of the CheW-like domains of all the *V. cholerae* CheV's with the *V. cholerae* and *E. coli* CheW proteins showed that these domains have the conserved arginine residue known to modulate CheA kinase activity. There is also significant charge and residue conservation within CheW regions responsible for chemoreceptor binding. These findings thus suggest that the *V. cholerae* CheV's likely retain CheW-like ability to interact with both chemoreceptors and CheA. Our results showing the polar localization of these CheV proteins similar to that of System II Che proteins and also the effect of CheV1 on swarming ability argue that CheV1 may be involved in the formation and/or regulation of the receptor-kinase complex.

IV. FUTURE PERSPECTIVES

To further validate that among the four CheV's, CheV1 is involved in chemotaxis, mutant strains carrying a deletion in each of the *cheV* genes need to be constructed. A more detailed study of the each CheW-like and CheY-like domain is required to explain the precise role and mechanism of the individual CheV proteins and to further our understanding in the chemotaxis of *V. cholerae*.

REFERENCES

- [1] J. P., "Armitage, bacterial tactic responses," *Adv Microb Physiol* vol. 41, pp. 229–289, 1999.
- [2] J. J. Falke, R. B. Bass, S. L. Butler, S. A. Chervitz, and M. A. Danielson, "The two-component signaling pathway of bacterial chemotaxis: a molecular view of signal transduction by receptors, kinases, and adaptation enzymes," *Annu Rev Cell Dev Biol*, vol. 13, pp. 457–512, 1997.
- [3] M. D. Manson, "Bacterial motility and chemotaxis," *Adv Microb Physiol*, vol. 33, pp. 277–346, 1992.
- [4] V. Sourjik and H. C. Berg, "Localization of components of the chemotaxis machinery of *Escherichia coli* using fluorescent protein fusions," *Mol. Microbiol*, vol. 37, pp. 740–751, 2000.
- [5] J. B. Stock and M. G. Surette, "Chemotaxis. In *Escherichia coli* and *Salmonella typhimurium*: Cellular and Molecular Biology," 2nd edn. F. C. Neidhardt, R. Curtiss III, J. J. Ingram, E. C. C. Lin, K. B. Low, B. Magasanik, et al. (eds). Washington, DC: American Society for Microbiology Press, vol. 1, pp. 1103–1129, 1996.
- [6] J. R. Maddock and L. Shapiro, "Polar location of the chemoreceptor complex in the *Escherichia coli* cell," *Science*, vol. 259, pp. 1717–1723, 1993.
- [7] M. M. L. Rosario, K. L. Frederick, G. W. Ordal, and J. D. Helmann, "Chemotaxis in *Bacillus subtilis* requires either of two functionally redundant CheW homologues," *J. Bacteriol*, vol. 176, pp. 2736–2739, 1994.
- [8] J. F. Heidelberg, J. A. Eisen, W. C. Nelson, R. A. Clayton, M. L. Gwinn, R. J. Dodson, D. H. Haft, E. K. Hickey, J. D. Peterson, L. Umayam, et al., *Nature*, vol. 406, pp. 477–483, 2000.
- [9] Z. Yang, Y. Geng, D. Xu, H. B. Kaplan, and W. Shi, "A new set of chemotaxis homologues is essential for *Myxococcus xanthus* social motility," *Mol. Microbiol*, vol. 30, pp. 1123–1130, 1998.
- [10] C. K. Stover, X. Q. Pham, A. L. Erwin, S. D. Mizoguchi, P. Warrener, M. J. Hickey, F. S. Brinkman, W. O. Hufnagle, D. J. Kowalik, M. Lagrou, R. L. Garber, L. Goltry, E. Tolentino, S. Westbrook-Wadman,

- Y. Yuan, L. L. Brody, S. N. Coulter, K. R. Folger, A. Kas, K. Larbig, R. Lim, K. Smith, D. Spencer, G. K. Wong, Z. Wu, and I. T. Paulse, "Complete genome sequence of *Pseudomonas aeruginosa* PA01, an opportunistic pathogen," *Nature*, vol. 406, pp. 947–948, 2000.
- [11] A. C. Martin, G. H. Wadhams, and J. P. Armitage, "The roles of the multiple CheW and CheA homologues in chemotaxis and in chemoreceptor localization in *Rhodobacter sphaeroides*," *Mol. Microbiol*, vol. 40, pp. 1261–1272, 2001.
- [12] K. K. Gosink, R. Kobayashi, I. Kawagishi, and C. C. Hase, *J. Bacteriol*, vol. 184, pp. 1767–1771, 2002.
- [13] A. Hyakutake, M. Homma, M. J. Austin, M. A. Boin, C. C. Hase, and I. Kawagishi, "Only one of the five CheY homologs in *Vibrio cholerae* directly switches flagellar rotation," *J. Bacteriol*, vol. 187, pp. 8403–10, 2005.
- [14] K. L. Fredrick and J. D. Helmann, "Dual chemotaxis signaling pathways in *Bacillus subtilis*: a UD-dependent gene encodes a novel protein with both CheW and CheY homologous domains," *J. Bacteriol*, vol. 176, pp. 2727–2735, 1994.
- [15] M. S. Pittman, M. Goodwin, and D. J. Kelly, "Chemotaxis in the human gastric pathogen *Helicobacter pylori*: different roles for CheW and the three CheV paralogues, and evidence for CheV2 phosphorylation," *Microbiology*, vol. 147, pp. 2493–2504, 2001.
- [16] R. A. Alm, L. S. L. Ling, D. T. Moir, and 20 other authors, "Genomic sequencing comparison of two unrelated isolates of the human gastric pathogen *Helicobacter pylori*," *Nature*, vol. 397, pp. 176–180, 1999.
- [17] J. F. Tomb, O. White, A. R. Kerlavage, and 39 other authors, "The complete genome sequence of the gastric pathogen *Helicobacter pylori*," *Nature*, vol. 388, pp. 539–547, 1997.
- [18] J. Frye, J. E. Karlinsey, H. Felise, B. Marzolf, N. Dowidar, M. McClelland, and K. T. Hughes, *J. Bacteriol*, vol. 188, pp. 2233–2243, 2006.
- [19] A. C. Lowenthal, C. Simon, A. S. Fair, K. Mehmood, K. Terry, S. Anastasia, and K. M. Ottemann, "A fixed-time diffusion analysis method determines that the three *cheV* genes of *Helicobacter pylori* differentially affect motility," *Microbiology*, vol. 155, pp. 1181–1191, 2009.
- [20] J. J. Mekalanos, D. J. Swartz, G. D. Pearson, N. Harfort, F. Groyne, and M. De Wilde, "Cholera toxin genes: nucleotide sequence, deletion analysis and vaccine development," *Nature*, vol. 306, no. 5943, pp. 551–7, 1983.
- [21] D. A. Sanders, B. Mendez, D. E. Koshland, "Role of the CheW protein in bacterial chemotaxis: overexpression is equivalent to absence," *J. Bacteriol*, vol. 171, pp. 6271–6278, 1989.

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