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NOTES

Analyses of the Roles of the Three *cheA* Homologs in Chemotaxis of *Vibrio cholerae*

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The *Vibrio cholerae* genome revealed the presence of multiple sets of chemotaxis genes, including three *cheA* gene homologs. We found that the *cheA*-2, but not *cheA*-1 or *cheA*-3, gene is essential for chemotaxis under standard conditions. Loss of chemotaxis had no effect on virulence factor expression in vitro.

Vibrio cholerae, the causative agent of cholera, is a gram-negative motile bacterium with a single polar flagellum. *V. cholerae* has a life cycle consisting of two distinct phases. Outside the host, the bacteria have a free-swimming phase, during which the major virulence factors are not expressed (for a review see reference 14). In the host, the bacteria enter a sessile virulent phase where the organisms adhere to the intestinal epithelium, replicate, and cause disease. Eventually the bacteria detach and exit the host via the profuse diarrhea that is the hallmark of cholera. Once back in the aquatic environment, the cycle can begin again.

Motility is an important virulence factor in many pathogenic species and in some cases is inversely regulated with the expression of virulence traits (12). Although the roles of motility and chemotaxis of *V. cholerae* in its ability to cause cholera has not been clearly established (3, 8), the production of the two major virulence factors, cholera toxin (CT) and toxin-coregulated pili (TCP), is known to be affected by the motility phenotype of the bacteria (3). Despite the potentially very important roles of chemotaxis in both the free-swimming as well as virulent phases of *V. cholerae*, no detailed genetic analysis of its chemotactic behavior has been performed. In the present study we generated several deletion mutants defective in putative chemotaxis genes and analyzed their motility behavior.

***V. cholerae cheA* genes.** The recently completed genome revealed several chemotaxis-related gene homologs in *V. cholerae* (7), most of which are clustered in three different regions distributed on both chromosomes (Fig. 1). Similarly, several other organisms, including *Pseudomonas aeruginosa* (13), *Rhodobacter sphaeroides* (9), *Myxococcus xanthus* (15), and *Borrelia burgdorferi* (2), have been reported to contain multiple chemotaxis operons. In *V. cholerae*, three putative genes with strong homology to the *Escherichia coli cheA* gene can be identified. The *cheA*-1 (VC1397) and *cheA*-2 (VC2063) genes are located on the larger chromosome of *V. cholerae*, whereas *cheA*-3 (VCA1095) is found on the smaller chromosome (7) (Fig. 1) and showed 38, 40, and 49% identity to the *E. coli cheA* gene, respectively.

Mutants in *cheA*. We created mutant strains carrying deletions in each of the *V. cholerae cheA* genes (VcheA-1, VcheA-2, VcheA-3) as well as a triple mutant strain (VcheA-123) by homologous recombination. Sequence data for *V. cholerae* were obtained from The Institute for Genomic Research website at http://www.tigr.org. The genes and surrounding sequences were amplified in PCRs by using specific primers and were cloned into plasmid vectors. Internal deletions were generated by using convenient restriction sites present in the genes or by cloning two PCR-derived DNA fragments containing the 5′- and 3′-flanking genomic regions adjacent to each other. The DNA fragments carrying the desired deletions were subcloned into the suicide vector pWM91 (10), and the mutated alleles were then introduced into the chromosome of the *V. cholerae* O395N1 strain following sucrose selection as described previously (1). The strains and plasmids used in this study are listed in Table 1. When assayed in 0.3% Luria-Bertani (LB) soft agar, the parental strain and the VcheA-1 and VcheA-3 mutant strains showed similarly large swarm circles, whereas the VcheA-2 and VcheA-123 strains showed very small swarm circles (Fig. 2). As expected, the *cheA* mutant derivative of an *E. coli* strain (EcheA) displays small swarm circles compared to that of its parent strain (Fig. 2). This indicates that the *cheA*-2 gene of *V. cholerae* is required for swarming in LB, consistent with previous results (8), whereas *cheA*-1 and *cheA*-3 are not. Similarly, in several other species only some of the multiple *che* genes appear to function in the chemotaxis behavior, including *R. sphaeroides* (9) and *M. xanthus* (15). It is possible that the multiple *che* genes serve as secondary chemotactic genes, and we have yet to discover the conditions under which these genes are needed, or they might function in other cellular processes such as development.

Complementation of *cheA*. Expression plasmids were generated by cloning the PCR products of the different *cheA* genes into the plasmid vector pBAD-TOPO (Invitrogen) via a cloning kit. All three *V. cholerae cheA* genes as well as the *E. coli cheA* gene were cloned under the control of an arabinose-

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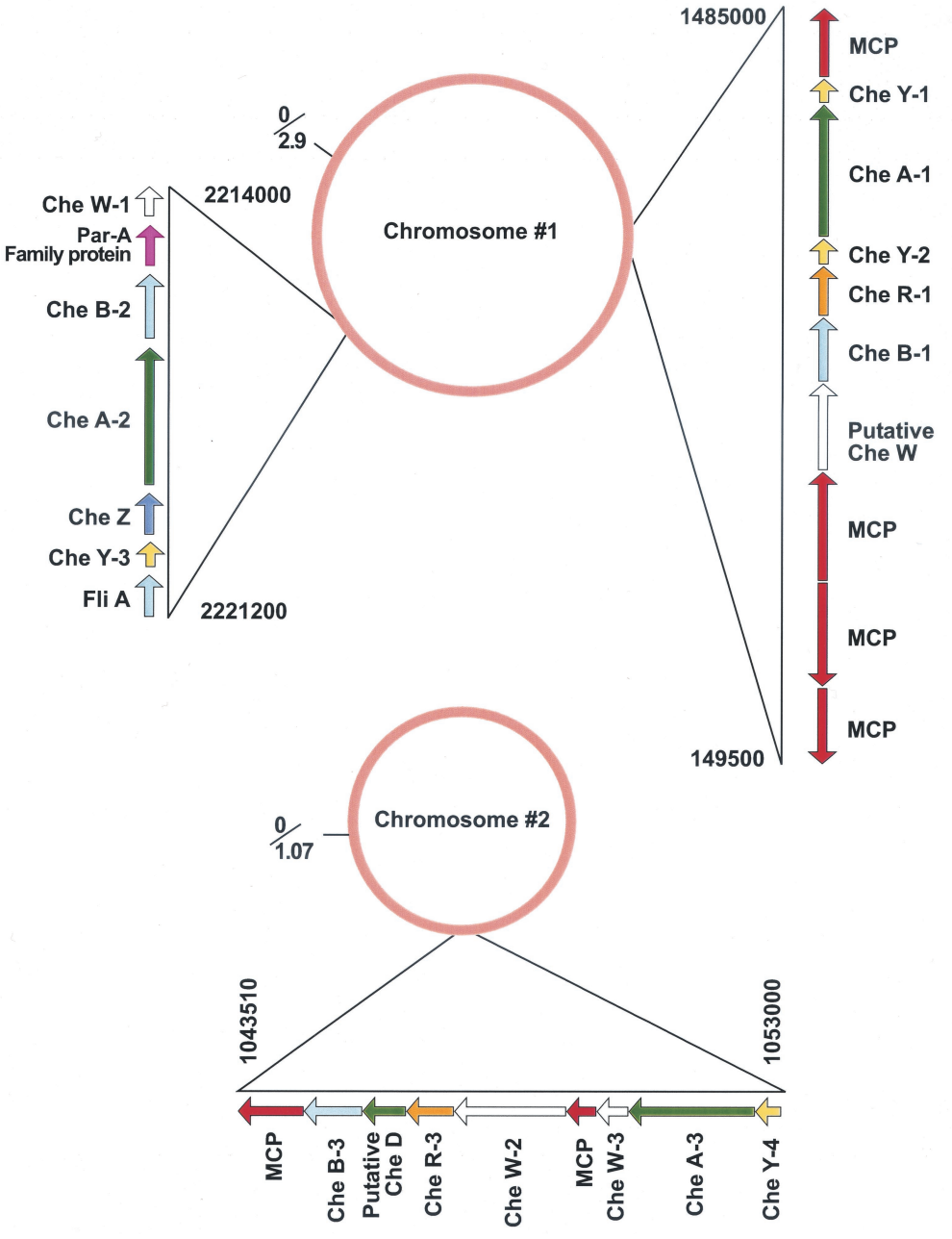

FIG. 1. Diagram of the location of the *cheA* genes in the two chromosomes of *V. cholerae*. The *cheA*-1 (VC1397) and *cheA*-2 (VC2063) genes are located on the larger chromosome of *V. cholerae*, whereas *cheA*-3 (VCA1095) is found on the smaller chromosome. MCP, methyl-accepting chemotaxis protein.

inducible promoter and were transformed into the VcheA-2, VcheA-123, and EcheA deletion strains by electroporation. As the VcheA-1 and VcheA-3 strains did not show any chemotaxis-deficient phenotype in this assay, we did not check it for complementation. We analyzed motility of the VcheA-2 (Fig. 3A) and VcheA-123 (data not shown) strains having plasmids that carry either of the three *V. cholerae cheA* genes or pEcheA by stabbing bacteria into LB soft agar. Both strains carrying pVcheA-2 showed marked increase in swarm circle size in the presence of 0.02% arabinose, whereas the presence of the *V. cholerae cheA*-1 or *cheA*-3 gene, even when expressed from an arabinose-inducible promoter, did not increase the swarm circle size in soft agar. However, induction of the *E. coli cheA* gene resulted in only very slightly larger swarm zones in both background strains compared to those of the empty plasmid control (Fig. 3A), which was more prominent after longer incubation periods (data not shown). As expected, the *E. coli cheA* deletion strain was complemented by the plasmid carrying the *E. coli cheA* gene (Fig. 3B) in the presence of the inducer. However, the *E. coli cheA* mutant strain was comple-

TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Relevant genotype or description	Source or reference
V. cholerae		
O395N1	Parental	J. J. Mekalanos
VcheA-1	O395N1 Δ*cheA*-1	This study
VcheA-2	O395N1 Δ*cheA*-2	This study
VcheA-3	O395N1 Δ*cheA*-3	This study
VcheA-123	O395N1 Δ*cheA*-1, Δ*cheA*-2, Δ*cheA*-3	This study
T:Z	O395N1 *toxT*::*lacZ*	6
T:ZcheA-1	O395N1 *toxT*::*lacZ* Δ*cheA*-1	This study
T:ZcheA-2	O395N1 *toxT*::*lacZ* Δ*cheA*-2	This study
T:ZcheA-3	O395N1 *toxT*::*lacZ* Δ*cheA*-3	This study
T:ZcheA-123	O395N1 *toxT*::*lacZ* Δ*cheA*-1, Δ*cheA*-2, Δ*cheA*-3	This study
TCP-2	O395 *tcpA*::Tn*phoA*	R. K. Taylor
TCP-2cheA-2	TCP-2 Δ*cheA*-2	This study
KP2.11	O395 *ctx*::*phoA*	R. K. Taylor
KP2.11cheA-2	KP2.11 Δ*cheA*-2	This study
E. coli		
RP437	Parental	J. S. Parkinson
EcheA	(RP9535) RP437 Δ*cheA*	J. S. Parkinson
Plasmids		
pBAD-TOPO	Expression vector	Invitrogen
pVcheA-1	pBAD-TOPO with *V. cholerae cheA*-1	This study
pVcheA-2	pBAD-TOPO with *V. cholerae cheA*-2	This study
pVcheA-3	pBAD-TOPO with *V. cholerae cheA*-3	This study
pEcheA	pBAD-TOPO with *E. coli cheA*	This study

mented well by pVcheA-2 but not by pVcheA-1 or pVcheA-3 (Fig. 3B). These data suggest that the *V. cholerae cheA*-2 gene encodes a functional homolog of the *E. coli* CheA protein. The *E. coli cheA* gene appears to partially compensate for the absence of the *V. cholerae cheA*-2 gene, whereas the *V. cholerae cheA*-2 gene can almost fully complement the *E. coli* deletion strain. Further experiments will be needed to determine the underlying mechanism of this difference.

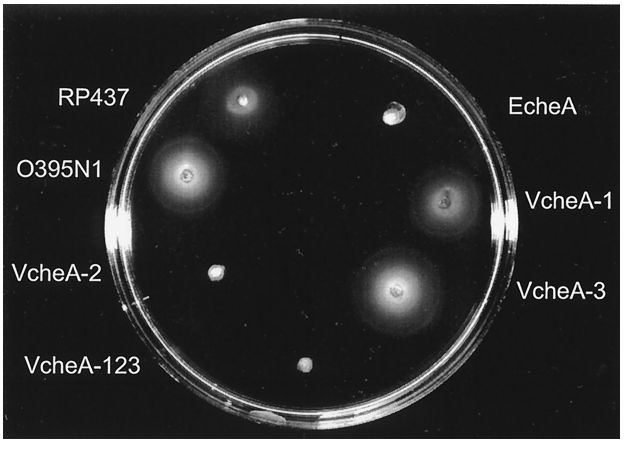

FIG. 2. Analyses of mutants for motility. Swarms in LB soft agar of the *V. cholerae* strain O395N1 (wild type) and it's *cheA*-1 (VcheA-1), *cheA*-2 (VcheA-2), *cheA*-3 (VcheA-3), and triple (VcheA-123) mutant derivatives as well as the *E. coli* strain RP437 and its *cheA* mutant derivative are shown. Plates were incubated for 8 h at 37°C.

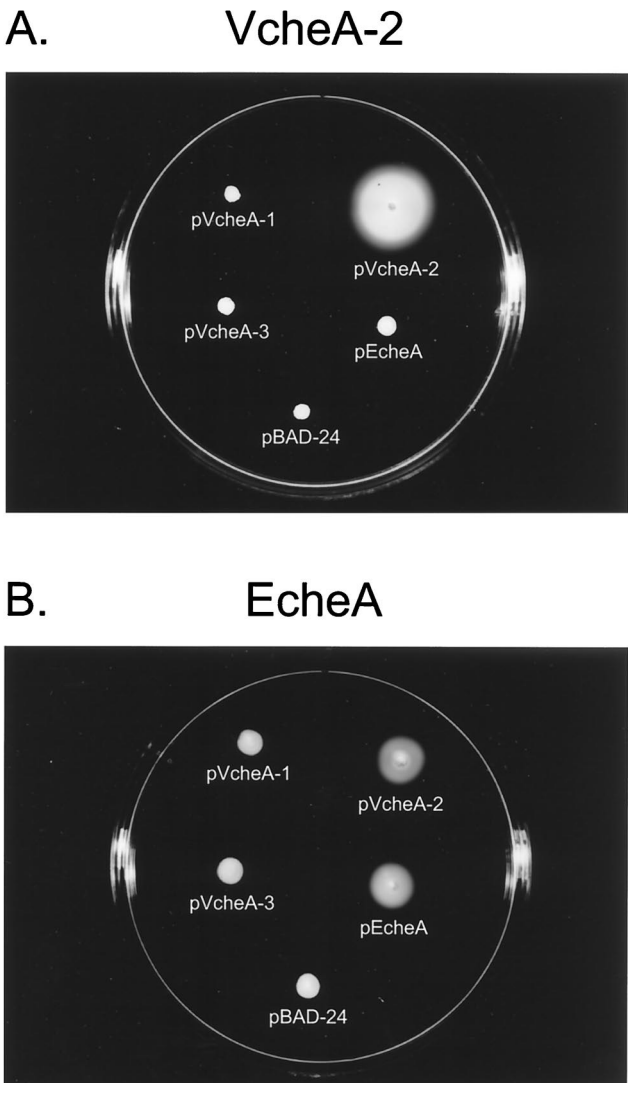

FIG. 3. Complementation of *cheA* mutants by plasmids carrying various *cheA* genes. (A) Swarming abilities in the presence of arabinose of the *V. cholerae cheA*-2 deletion strain (VcheA-2) complemented by plasmids carrying the *V. cholerae cheA*-1 (pVcheA-1), *cheA*-2 (pVcheA-2), or *cheA*-3 (pCheA-3) gene or the *E. coli cheA* gene. (B) Swarming abilities in the presence of arabinose of the *E. coli cheA* deletion strain (EcheA) complemented by plasmids carrying the *V. cholerae cheA*-1 (pVcheA-1), *cheA*-2 (pVcheA-2), or *cheA*-3 (pCheA-3) gene or the *E. coli cheA* gene. pBAD-24 is the parent vector and contains no *cheA* gene. Plates were incubated for 8 h at 37°C.

Detection of the CheA proteins in *V. cholerae* cells. Expression of the CheA proteins was examined. Bacterial whole-cell extracts were subjected to immunoblotting with anti-*E. coli* CheA serum. Fresh overnight cultures were diluted 1:30 into fresh Tryptone-glycerol medium supplemented with or without arabinose. Cells were grown at 37°C for 3 h with vigorous shaking, harvested by centrifugation, and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis followed by immunoblotting. Immunoblotting was performed, essentially as described previously (11), with rabbit anti-*E. coli* CheA

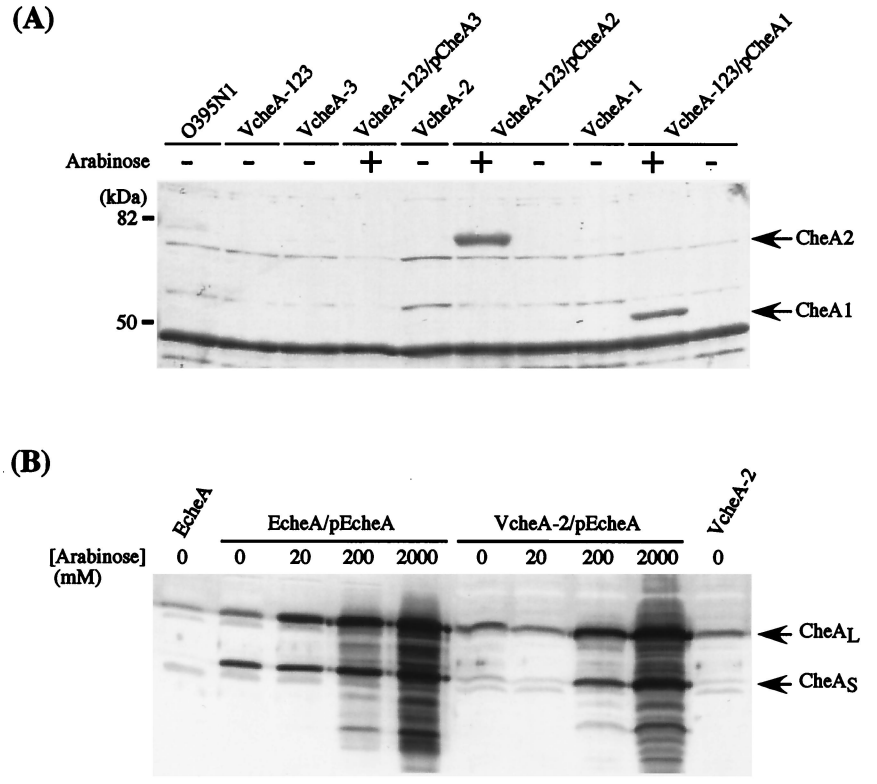

FIG. 4. Detection of the CheA proteins in *V. cholerae* and *E. coli* cells. (A) Cultures were grown with (+) or without (−) 20 mM arabinose. The arrows indicate the positions of the bands corresponding to the CheA-1 and CheA-2 proteins. (B) The amount of arabinose added to the cultures is indicated above the lanes. The arrows indicate the positions of the bands corresponding to the large ($CheA_L$) and small ($CheA_S$) forms of the *E. coli* CheA protein. Immunoblottings were performed with rabbit anti-*E. coli* CheA serum as the first antibody and alkaline phosphatase-conjugated goat anti-rabbit immunoglobulin G antibody as the second antibody. The plasmids introduced into the strains are indicated after a slash.

serum as the first antibody and alkaline phosphatase-conjugated goat anti-rabbit immunoglobulin G antibody as the second antibody. Although a number of bands were detected for O395N1 cells, all of them were also detected for its derivative VcheA-123, suggesting that none of them are related to CheA (Fig. 4A). However, unique bands corresponding to CheA-1 or CheA-2 were detected for VcheA-123 cells carrying pCheA-1 or pCheA-2 in the presence of, but not in the absence of, arabinose, suggesting that these proteins were overexpressed by the induction of the *araBAD* promoter (Fig. 4A). In contrast, no band corresponding to CheA-3 was detected for VcheA-123 cells carrying pCheA-3, even in the presence of arabinose. Expression of the *E. coli* CheA protein was confirmed in both the *E. coli* and *V. cholerae cheA* deletion strains in response to the addition of arabinose (Fig. 4B).

Virulence gene expression. Random nonmotile mutants of *V. cholerae* showed increased expression of the essential virulence factors, CT and TCP (3), and we recently reported elevated levels of *toxT*::*lacZ* expression in defined nonmotile strains (5). Furthermore, at least two ToxR-regulated genes encode proteins with homology to methyl-accepting chemotaxis proteins, and one of them, *tcpI*, has been suggested to be involved in virulence gene regulation (4). This prompted us to analyze the effects of the different *V. cholerae cheA* mutations on the expression of *toxT*. Single mutations in the three *cheA* genes as well as triple mutations were introduced into the *V. cholerae* O395N1 *toxT*::*lacZ* reporter strain previously described (6). Similar β-galactosidase levels of the mutant strains compared to those of the parental strain were observed (data not shown). Further, we introduced deletions in the *cheA*-2 genes into *V. cholerae* strains carrying *ctx*::*phoA* or *tcpA*::*phoA* reporter constructs and observed no differences in alkaline phosphotase expression levels between the parental and chemotaxis-deficient strains (data not shown), indicating no direct link between lack of chemotaxis and virulence gene regulation in *V. cholerae* in vitro. Interestingly, Lee et al. recently reported that several *V. cholerae* chemotaxis genes, including *cheA*-2, regulate virulence gene expression in an in vivo model (8).

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