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Only One of the Five CheY Homologs in *Vibrio cholerae* Directly Switches Flagellar Rotation

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Vibrio cholerae **has three sets of chemotaxis (Che) proteins, including three histidine kinases (CheA) and four response regulators (CheY) that are encoded by three *che* gene clusters. We deleted the *cheY* genes individually or in combination and found that only the *cheY3* deletion impaired chemotaxis, reinforcing the previous conclusion that *che* cluster II is involved in chemotaxis. However, this does not exclude the involvement of the other clusters in chemotaxis. In other bacteria, phospho-CheY binds directly to the flagellar motor to modulate its rotation, and CheY overexpression, even without CheA, causes extremely biased swimming behavior. We reasoned that a *V. cholerae* CheY homolog, if it directly controls flagellar rotation, should also induce extreme swimming behavior when overproduced. This was the case for CheY3 (*che* cluster II). However, no other CheY homolog, including the putative CheY (CheY0) protein encoded outside the *che* clusters, affected swimming, demonstrating that these CheY homologs cannot act directly on the flagellar motor. CheY4 very slightly enhanced the spreading of an *Escherichia coli cheZ* mutant in semisolid agar, raising the possibility that it can affect chemotaxis by removing a phosphoryl group from CheY3. We also found that *V. cholerae* CheY3 and *E. coli* CheY are only partially exchangeable. Mutagenic analyses suggested that this may come from coevolution of the interacting pair of proteins, CheY and the motor protein FliM. Taken together, it is likely that the principal roles of *che* clusters I and III as well as *cheY0* are to control functions other than chemotaxis.**

Vibrio cholerae, the causative agent of cholera, inhabits river, estuary, and other aquatic environments, where it actively swims by rotating its polar flagellum as a screw-like propeller. When introduced into a host with contaminated foods or water, the bacterium reduces its swimming motility and colonizes the epithelium of the small intestine, where it produces cholera toxin (CT), which causes severe diarrhea, a diagnostic symptom of cholera (27). It has been reported that some chemotaxis-related genes are involved in the pathogenicity of *V. cholerae* (1, 3, 9, 15, 20). A recent study showed that some nonchemotactic mutants outcompete the wild-type strain during infection (6, 7). Thus, studying the mechanisms of motility and chemotaxis is of vital importance to understanding the pathogenicity and survival strategies of this bacterium.

Signal transduction in chemotaxis, which involves control of flagellar rotation in response to environmental stimuli, has been best studied with *Escherichia coli* (for reviews, see references 2, 10, and 32). Central to this pathway is a two-component regulatory system that consists of the histidine kinase CheA and the response regulator CheY. CheA is activated when coupled to an unliganded chemoreceptor (also known as methyl-accepting chemotaxis protein [MCP]). CheA phosphorylates itself and transfers a phosphate group to CheY. Binding of phospho-CheY to the switch complex of the flagellar motor, which otherwise rotates counterclockwise, induces its clockwise (CW) rotation, resulting in reorientation of the cell and hence an abrupt change in its swimming direction.

The genome sequence of *V. cholerae* El Tor predicts that the bacterium has three sets of Che proteins and 45 MCP-like proteins (hereafter called MLPs) (16). Each set of *che* genes forms clusters as follows: *che* cluster I contains *cheY1* (VC1395), *cheA1* (VC1397), *cheY2* (VC1398), *cheR1* (VC1399), *cheB1* (VC1401), and the putative gene *cheW* (VC1402); cluster II contains *cheW1* (VC2059), *cheB2* (VC2062), *cheA2* (VC2063), *cheZ* (VC2064), and *cheY3* (VC2065); and cluster III contains *cheB3* (VCA1089), *cheD* (VCA1090), *cheR3* (VCA1091), *cheW2* (VCA1093), *cheW3* (VCA1094), *cheA3* (VCA1095), and *cheY4* (VCA1096). *cheR2* (VC2201) is in a *fla* gene cluster located just next to *che* cluster II. Clusters I and II are located on chromosome I, and cluster III is located on chromosome II. In addition, chromosome I contains a pair of genes encoding a non-CheA histidine kinase (VC1315) and a putative CheY protein (VC1316) outside of any *che* or *fla* gene cluster.

Several lines of evidence suggest that at least some of these chemotaxis-related genes are involved in virulence. An in vivo screen and further characterization revealed that several chemotaxis genes (*mcpX* [VC2161], *cheZ*, *cheA2*, and *cheY3*) are required for the production of CT or its positive regulator ToxT upon infection of mice, although the production of CT in vitro does not require any of these genes except *cheZ* (20). Nonchemotactic mutants with counterclockwise- and clockwise-biased flagellar rotation were reported to show enhanced and attenuated infectivity, respectively (6). In an in vitro study (3), cells lacking or overproducing CheY4 exhibited a decreased or increased production of CT, respectively, and CheY4

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was suggested to be involved in attachment to host cell layers and migration toward ingredients of the intestine. It was also reported that two MLPs (TcpI and AcfB) are involved in colonization (9, 15) and that another MLP (HlyB) is involved in the secretion of hemolysin (1, 18).

In this context, it is important to examine whether only one *che* cluster is involved in the chemotaxis of *V. cholerae*, as suggested earlier (12). The deletion of *cheA2*, but not *cheA1* or *cheA3*, abolishes chemotaxis, and only *cheA2* complements the deletion of *cheA2*. A deletion of *cheY3* was also reported to impair chemotaxis (6, 7). These results demonstrate that cluster II containing *cheA2* and *cheY3* is involved in chemotaxis. However, the results do not exclude the possible involvement of clusters I and III in chemotaxis, since the *che* genes of these clusters (or any other genes required for the functions of the *cheA* genes) might not be expressed sufficiently under the conditions tested.

In this study, we focused on the *V. cholerae* CheY homologs that are predicted from the genome sequence. Overproduction of the *E. coli* CheY protein ($CheY_E$) induces CW rotation of flagella even in a gutted *E. coli* strain, which is deleted for *cheA* and all other *che* genes. The D13K mutation of $CheY_E$ induces CW flagellar rotation without involving phosphorylation (5). In *Bacillus subtilis*, the situation is a little different: the overproduction of CheY ($CheY_B$) causes incessant smooth swimming, and the corresponding D-to-K mutation fails to activate $CheY_B$ (4). We therefore reasoned that the overproduction of any of the CheY homologs that are involved in the chemotaxis of *V. cholerae* should induce biased flagellar rotation and extreme swimming behavior in *V. cholerae*. We generated several specific deletion mutants, cloned all of the predicted *cheY* genes from *V. cholerae*, and substituted Lys for the aspartate residues corresponding to Asp-13 of $CheY_E$. We then examined the effects of these wild-type or DK mutant CheY proteins on the swimming behavior of *V. cholerae* as well as *E. coli*. Among all five *V. cholerae* CheY proteins, only CheY3 affected chemotaxis, suggesting that this is the only CheY protein directly controlling flagellar rotation. Among the other CheY homologs, CheY4 very slightly improved spreading in semisolid agar of an *E. coli cheZ* mutant strain, raising the possibility that it can indirectly affect chemotaxis by receiving a phosphoryl group from CheY3.

MATERIALS AND METHODS

Bacterial strains. *V. cholerae* classical strain O395N1 has wild-type chemotaxis. Strain VcheA123 (12), derived from O395N1, lacks all three *cheA* genes. The O395N1 derivative strains VcheY1, VcheY2, VcheY3, and VcheY4, each of which has one *cheY* gene deleted, were constructed as described below. All of the *E. coli* strains used are derivatives of K-12. Strain RP437 has wild-type chemotaxis (25). Strain HCB1262 [Δ(*cheA-Z*)::*zeoR thr leu his met rpsL136*], lacking all *che* genes, *tar*, and *tap*, was used as a nontumbling strain (H. C. Berg, personal communication). Strain RP1616, lacking *cheZ*, was used as a constantly tumbling strain (J. S. Parkinson, personal communication).

Plasmids. The vector pFLAG-CTC (Sigma), derived from plasmid pBR322, carries the *lacI* and *bla* genes and encodes a FLAG M2 tag sequence downstream of the *tac* promoter. Plasmid pBAD33 (14), containing the p15A replicon and the *araBAD* promoter, is compatible with pFLAG-CTC in the same cell. The suicide vector pWM91 (22), containing the R6K origin but lacking the *pir* gene required for replication and the *sacB* genes required for sucrose counterselection, was used to disrupt the *cheY* genes by homologous recombination.

Cloning of *cheY* genes. Each *cheY* gene was amplified by PCR, with chromosomal DNA of *V. cholerae* strain O395N1 or *E. coli* strain W3110 as the template, to introduce XhoI and KpnI sites at the 5′ and 3′ ends of the coding region, respectively. The resulting DNA fragments were digested with XhoI and KpnI and cloned into the vector plasmid pFLAG-CTC so that the FLAG tag was fused to the carboxyl terminus of each CheY protein.

The D-to-K mutations of the CheY homologs corresponding to the constitutively active D13K mutation of $CheY_E$ were introduced by site-directed mutagenesis using mutant forward primers. The charge-reversing mutations of $CheY_E$ and CheY3 were introduced similarly, using mutant reverse primers. The D58K mutant of CheY4 was constructed by a two-step PCR method as described previously (34). The resulting fragments of the mutant *cheY* genes were cloned into pFLAG-CTC.

The fragment encoding CheY1-FLAG was amplified by PCR to introduce a SacI site and a Shine-Dalgarno (SD) sequence at the 5′ end and a PstI site at the 3′ end of the fragment. The resulting DNA fragment was digested with SacI and PstI and cloned into pBAD33.

Deletion of *cheY* genes. In-frame deletions in the *cheY* genes were constructed by overlap extension PCR (17, 35), using four primers for each gene. A region of DNA from the *V. cholerae* chromosome spanning from approximately 1 kb upstream to the 5′ end of the desired gene was PCR amplified using primers 1 and 2. A second region, from approximately 1 kb downstream to the 3′ end of the gene, was PCR amplified using primers 3 (complementary to a portion of primer 2) and 4. A mixture of these two DNA fragments (100 ng each) was used as the template in a third PCR amplification using primers 1 and 4. The product of the third amplification contained an approximately 2-kb fragment with an in-frame deletion in the desired gene, with engineered restriction sites at its 5′ and 3′ ends that allowed subcloning of the DNA fragment into the suicide vector pWM91. A protocol for introducing cloned mutations into the chromosome of *V. cholerae* by homologous recombination using a suicide vector system followed by sucrose selection was used (8).

Nucleotide sequencing. Nucleotide sequences of the cloned genes were determined by the dideoxy chain termination method, using a BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems).

Analyses of amino acid sequences and three-dimensional structures of proteins. Amino acid sequences were aligned using Clustal W, version 1.4. Sequence homology and similarity were calculated using the BLAST 2 sequence program (33). Three-dimensional structures of proteins were drawn and analyzed by using Swiss PDB Viewer, version 3.7b1.

Immunoblotting. Immunoblotting was carried out essentially as described previously (24). An overnight culture of *E. coli* in TG (1% tryptone, 0.5% NaCl, 0.5% glycerol) was diluted 1:40 in fresh TG supplemented with IPTG (isopropyl-β-D-thiogalactopyranoside), if necessary. Cells were grown with vigorous shaking, harvested at late exponential phase, and washed with MLM buffer (10 mM potassium phosphate [pH 7.0], 0.1 mM EDTA, 10 mM sodium lactate, 100 μM methionine). An overnight culture of *V. cholerae* in TG was diluted 1:100 in fresh TG supplemented with IPTG, if necessary. Cells were grown with vigorous shaking, harvested at late exponential phase, and washed with TM buffer (50 mM Tris-HCl [pH 7.4], 5 mM glucose, 5 mM $MgCl_2$). The samples were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE; 15% polyacrylamide) followed by immunoblotting with anti-FLAG M2 antibody (Sigma).

Swarm assay. Swarm assays were performed using tryptone semisolid agar (1% tryptone, 0.5% NaCl, and 0.3% agar). When necessary, 50 μg ml^{-1} ampicillin and/or 0.1 or 1 mM IPTG was added. An aliquot (1 μl) of overnight culture in TG was spotted onto a plate, which was then incubated at 30°C for 6 to 24 h.

Observation of swimming behavior. Free-swimming cells were observed essentially as described previously (19, 23). Cells were grown as described above. *V. cholerae* cells were resuspended in TMN (50 mM Tris-HCl [pH 7.4], 5 mM glucose, 5 mM $MgCl_2$, 100 mM NaCl), and *E. coli* cells were resuspended in MLM buffer. After incubation at room temperature for 1 h, cells were observed under a dark-field microscope (Nikon 74257). Swimming tracks of the cells were traced using an Argus-10 image processor (Hamamatsu Photonics). The reorientation frequency of *V. cholerae* cells was defined as the number of changes of swimming direction per second and was calculated from the swimming tracks of 20 cells. The smooth-swimming fraction of *E. coli* was defined as the percentage of smooth-swimming cells (i.e., cells running without reorientation for 2 s) among the total swimming cells (>100 cells), not including cells attached to the glass surface.

Nucleotide sequence accession numbers. The sequence data for the *cheY* homologs of O395N1 have been deposited in the DDBJ database under the following accession numbers: *cheY0*, AB185929; *cheY1*, AB185930; *cheY2*, AB185931; *cheY3*, AB185932; and *cheY4*, AB185933.

FIG. 1. Sequence alignment of CheY homologs of *V. cholerae* with $CheY_E$. Amino acid sequences of the CheY homologs deduced from the DNA sequences of the cloned genes from *V. cholerae* O395N1 were aligned using Clustal W (version 1.4). CheY0 indicates the putative CheY homolog used throughout this paper. Particularly important residues that have been identified in CheY of *E. coli* ($CheY_E$) are marked with boxes with the following colors: black, phosphorylation site; white, sites for function as a response regulator (36); gray, sites interacting with FliM (21, 28, 30). Although the *cheY1* gene of *V. cholerae* El Tor biotype strain N16961 is deduced to encode a truncated product (up to Glu-94 of the product deduced from the database [the corresponding residue of the deduced product from the cloned gene is indicated by an arrow], with nine foreign residues) because of a frameshift, the *cheY1* gene of the *V. cholerae* classical biotype strain O395N1 was deduced to encode a full-length protein.

RESULTS

Cloning of the five *cheY* homologs of a classical *V. cholerae* strain and alignment of the deduced amino acid sequences of their products with that of $CheY_E$. The five *V. cholerae cheY* genes were cloned and sequenced as described in Materials and Methods. The deduced amino acid sequences of the different CheY homologs were aligned using Clustal W, version 1.4 (Fig. 1). The published genome sequence of the *V. cholerae* El Tor biotype strain predicts that its CheY1 protein is truncated because of a frameshift in the coding region. However, the *cheY1* gene cloned from the chromosome of *V. cholerae* classical biotype strain O395N1 had an insertion of a guanine nucleotide between guanine 282 and cytosine 283 of the published *cheY1* sequence of El Tor strain N16961, leading to retention of the full-length reading frame (encoding 126 amino acid residues).

The deduced amino acid sequences of the *V. cholerae* CheY0, CheY1, CheY2, CheY3, and CheY4 proteins showed 26%, 38%, 33%, 57%, and 36% identity and 42%, 57%, 52%, 74%, and 53% similarity, respectively, to that of $CheY_E$. The site of phosphorylation (Asp-57) and the other residues critical for phosphorylation (Asp-12, Asp-13, and Lys-109) in $CheY_E$ (36) were conserved perfectly in all of the *V. cholerae* CheY homologs (Fig. 1). The FliM-interacting residues were identified by characterizations of suppressors (28, 30) and by X-ray crystallography of a complex of $CheY_E$ and the N-terminal fragment of FliM (21). These FliM-interacting residues were best conserved in CheY3.

Detection of *cheY* gene products. The substitution of Lys for Asp-13 of $CheY_E$ results in a constitutively active phenotype, inducing CW rotation of the flagellar motor independent of phosphorylation (5). In addition, the corresponding D11K mutant of CheB also exhibits a constitutively active phenotype (31). Similar D-to-K mutants of the *V. cholerae* CheY proteins corresponding to D13K of $CheY_E$ were constructed to examine whether they induce extreme swimming behavior (Fig. 2A). The five *cheY* coding regions of *V. cholerae* O395N1 were cloned into pFLAG-CTC so that a FLAG peptide was fused to the carboxyl terminus of each CheY protein (Fig. 2A). The D-to-K mutations were introduced into the cloned genes, and the nucleotide sequences of all constructs were determined. The resulting plasmids encoding the wild-type or mutant version of each CheY-FLAG protein were introduced into *V. cholerae* O395N1 (Che^+) cells. The CheY-FLAG proteins were induced in *V. cholerae* with 1 mM IPTG and were detected by immunoblotting using anti-FLAG M2 antibody (Fig. 2B). All of the wild-type and mutant CheY-FLAG proteins were expressed at similar levels, except the wild-type and mutant CheY0-FLAG proteins, which were expressed at slightly higher levels. In the absence of IPTG, no CheY-FLAG protein was detected. In *E. coli* cells, all of the CheY-FLAG proteins were expressed at similar levels, except for the CheY0-FLAG proteins, which were expressed at slightly higher levels (data not shown).

Construction and characterization of *cheY* deletion strains. In-frame deletion mutants with deletions of the *V. cholerae*

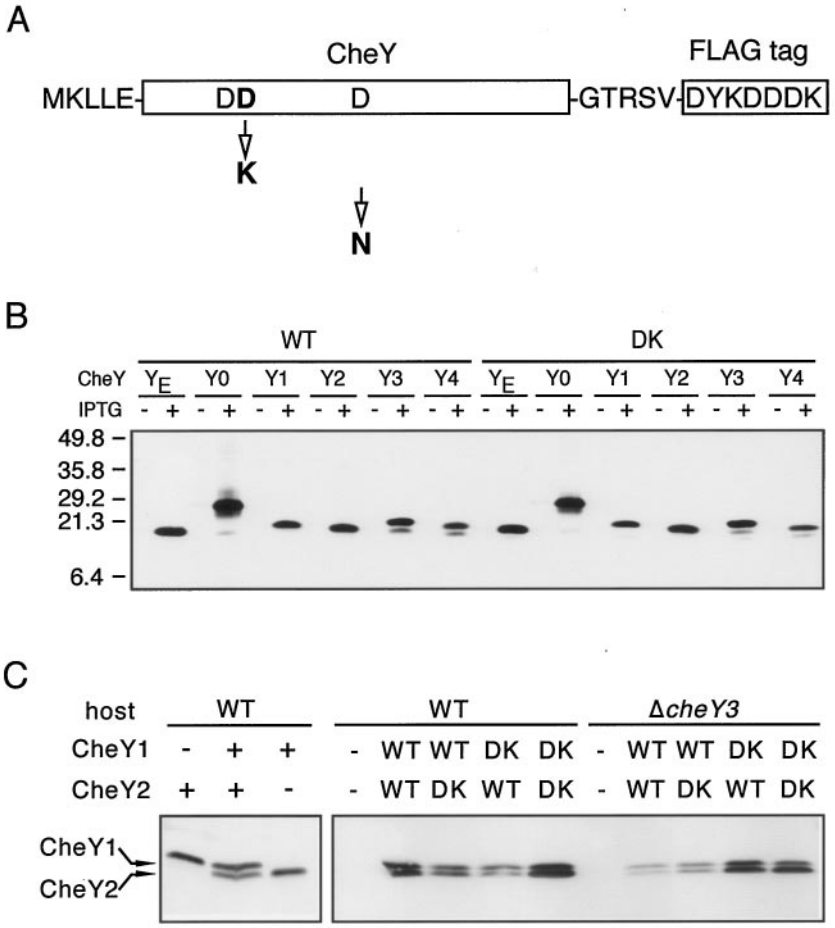

FIG. 2. Immunoblotting of CheY-FLAG proteins. (A) Schematic diagram of the CheY-FLAG proteins constructed for this study. The *V. cholerae cheY* coding regions were cloned into plasmid pFLAG-CTC so that the FLAG tag was fused to the carboxyl terminus of each CheY protein (wild type [WT]). The "MKLLE" and "GTRSV" sequences were derived from the vector. A D-to-K mutant of each CheY-FLAG protein, corresponding to the constitutively active D13K mutant of $CheY_E$, and a dominant negative mutant of CheY4-FLAG, corresponding to the nonphosphorylatable D57N mutant of $CheY_E$, were also constructed. (B) Expression of CheY-FLAG proteins in *V. cholerae*. The constructed plasmids were introduced into the Che^+ strain O395N1. Lysates of cells grown in the absence (−) or presence (+) of 1 mM IPTG were subjected to SDS-PAGE (15%) followed by immunoblotting with anti-FLAG M2 antibody. (C) Coexpression of CheY1 and CheY2-FLAG proteins in *V. cholerae*. The constructed plasmids were introduced into the Che^+ strain O395N1. Lysates of cells grown in the presence of 1 mM IPTG and 5 mM arabinose were subjected to SDS-PAGE (15%) followed by immunoblotting with anti-FLAG M2 antibody.

O395N1 *cheY1*, *cheY2*, *cheY3*, and *cheY4* genes were constructed, and the resulting strains were tested for chemotaxis. Only the Δ*cheY3* strain was defective in chemotactic spreading in semisolid agar (Table 1) and in reorientation in liquid medium (Fig. 3A). The phenotype of the Δ*cheY3* strain is consistent with those of previously reported *cheY3* mutant strains derived from the El Tor strain (6, 20). The single and multiple *cheY* deletion mutations in *cheY1*, *cheY2*, and *cheY4* did not affect swimming behavior (Fig. 3A; Table 1). Among the five *cheY* homologs, only the *cheY3* gene complemented the defects in the *cheY3* strain in spreading (Table 2) and reorientation (data not shown). To analyze the effect of coexpression of CheY1 and CheY2, we constructed pBAD33-based plasmids encoding the wild-type or D-to-K mutant version of CheY1-FLAG that are compatible with pCTC-FLAG and its derivatives. The coexpression of CheY1 and CheY2 (Fig. 2C) did not complement the defects of the *cheY3* mutant, either (data not shown). The $cheY_E$ gene only partially complemented defects

TABLE 1. Spreading in semisolid agar of *V. cholerae* cells lacking single or multiple *cheY* genes

Strain	Relative diameter[a]
Wild type	1.0 ± 0.14
Δ*cheY1*	1.1 ± 0.09
Δ*cheY2*	0.96 ± 0.07
Δ*cheY3*	0.14 ± 0.02
Δ*cheY4*	0.98 ± 0.04
Δ*cheY12*	1.01 ± 0.02
Δ*cheY14*	1.01 ± 0.02
Δ*cheY24*	0.98 ± 0.02
Δ*cheY124*	1.01 ± 0.02

[a] Overnight cultures were spotted onto tryptone–semisolid 0.3% agar, which was then incubated at 30°C for 7 h, and the diameters of the swarm rings were measured. The relative diameter of a swarm ring of a particular strain was defined as the ratio of the diameter to that of the wild-type strain. The average values with standard deviations from a triplicate assay are shown.

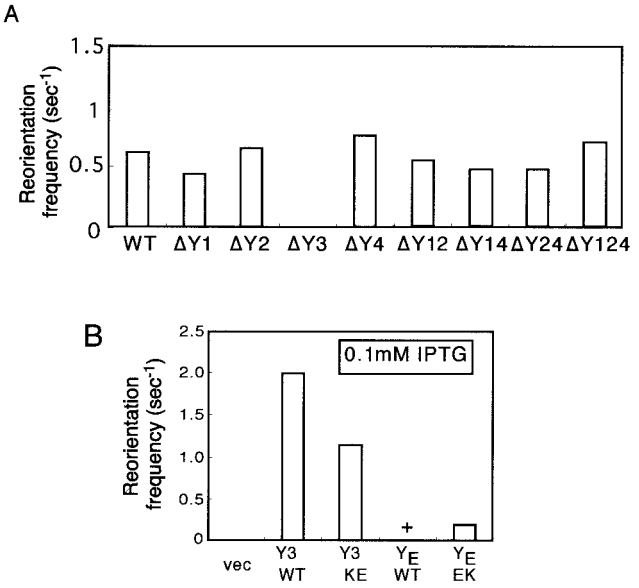

FIG. 3. Effects of CheY homologs on free swimming of *V. cholerae* cells in liquid medium. (A) Reorientation frequencies of wild-type and *cheY* knockout strains. Overnight cultures of cells in LB containing ampicillin (50 μg ml^{-1}) were diluted in fresh medium and grown with vigorous shaking. The cells were harvested at late logarithmic phase, washed, resuspended in TMN at room temperature, and observed under a dark-field microscope. The average reorientation frequency (s^{-1}) of each strain was determined by analyzing swimming tracks. (B) Reorientation of CheY-expressing VcheY3 (Δ*cheY3*) strain. VcheY3 cells carrying plasmids encoding wild-type or mutant CheY3 or $CheY_E$ were grown in the presence of 0.1 mM IPTG. Cells were observed and analyzed as described above. +, frequency is above zero.

of the *cheY3* mutant strain VcheY-3 in spreading (Table 2) and reorientation (<0.5 s^{-1}).

Effects of CheY homologs on swimming behavior of chemotactic *V. cholerae* cells. To determine whether any CheY homolog other than CheY3 can act directly on the flagellar motor, we examined the effects of overproduction of the CheY homologs in chemotactic (Che$^+$) *V. cholerae* cells (O395N1) (Table 3). Cells overexpressing wild-type or mutant CheY3 spread much slower than cells carrying the vector. The wild-type and mutant $CheY_E$ proteins had milder effects than the CheY3 proteins. The expression of any other *V. cholerae* CheY homolog had no significant effect. Free-swimming cells expressing wild-type or mutant CheY3 reoriented more frequently than cells carrying the vector (Fig. 4A). Cells expressing the D-to-K version of CheY3 reoriented more frequently than those expressing the wild-type version, indicating that the former version is more active than the latter in *V. cholerae* cells (Fig. 4A). The coexpression of CheY1 and CheY2 (Fig. 2C) did not affect spreading in semisolid agar (data not shown) or the reorientation frequency (Fig. 4B). A triple *cheY* mutant strain lacking *cheY1*, *cheY2*, and *cheY4* was also used to exclude any possible interaction between the CheY homologs that might suppress the effect of a particular CheY homolog, but again no CheY homolog other than CheY3 showed any detectable effect on spreading or swimming when overexpressed (data not shown). These results suggest that only CheY3 of the five *V. cholerae* CheY homologs can control the flagellar motor

TABLE 2. Spreading in semisolid agar of *V. cholerae* Δ*cheY3* cells expressing each CheY homolog

CheY homolog	Mutation	Relative diameter[a]
None	NA[b]	0.18 ± 0.02
$CheY_E$	None	0.43 ± 0.05
	D13K	0.33 ± 0.11
CheY0	None	0.18 ± 0.01
	D11K	0.15 ± 0.02
CheY1	None	0.17 ± 0.00
	D10K	0.17 ± 0.01
CheY2	None	0.20 ± 0.01
	D10K	0.17 ± 0.01
CheY3	None	1.0 ± 0.05
	D16K	1.08 ± 0.06
CheY4	None	0.20 ± 0.03
	D15K	0.17 ± 0.01
$CheY_E$	E117K	1.37 ± 0.03

[a] The relative diameter of a swarm ring was measured as described in Table 1, except that the average diameter of the triplicate swarm rings of wild-type cells expressing CheY3 was defined as 1.0, tryptone–semisolid 0.3% agar was supplemented with 0.1 mM IPTG, and the incubation time at 30°C was 12 h.
[b] NA, not applicable.

and that the D-to-K substitution in CheY3, like the corresponding mutation in $CheY_E$, causes a constitutively active phenotype.

Effects of CheY homologs on swimming behavior of *V. cholerae* cells lacking any CheA homolog. To exclude any possible involvement of the three CheA homologs encoded by *V. cholerae*, we examined the effects of the CheY homologs on the swimming behavior of VcheA123 cells, a *V. cholerae* derivative of O395N1 lacking all three *cheA* genes (12). Although nonchemotactic (Che$^-$) cells cannot form a chemotactic ring in semisolid agar, those with an unregulated but appropriate run/reorientation bias can spread significantly, albeit very slowly (37). Cells expressing wild-type or mutant CheY3 spread significantly in tryptone semisolid agar, whereas no other CheY homolog induced spreading (Table 4). Free-swimming cells expressing each CheY protein were also observed by microscopy, and only cells expressing wild-type or mutant CheY3 reoriented. Cells expressing the D-to-K version reori-

TABLE 3. Spreading in semisolid agar of wild-type *V. cholerae* cells expressing each CheY homolog

CheY homolog	Mutation	Relative diameter[a]
None	NA[b]	1.0 ± 0.03
$CheY_E$	None	0.67 ± 0.03
	D13K	0.45 ± 0.05
CheY0	None	0.84 ± 0.05
	D11K	0.88 ± 0.05
CheY1	None	0.96 ± 0.03
	D10K	0.88 ± 0.02
CheY2	None	1.06 ± 0.03
	D10K	0.96 ± 0.07
CheY3	None	0.55 ± 0.03
	D16K	0.42 ± 0.02
CheY4	None	1.00 ± 0.07
	D15K	0.92 ± 0.03

[a] The relative diameters of swarm rings were measured as described in Table 1, except that the average diameter of the triplicate swarm rings of wild-type cells with the vector was defined as 1.0, tryptone–semisolid 0.3% agar was supplemented with 0.1 mM IPTG, and the incubation time at 30°C was 6 h.
[b] NA, not applicable.

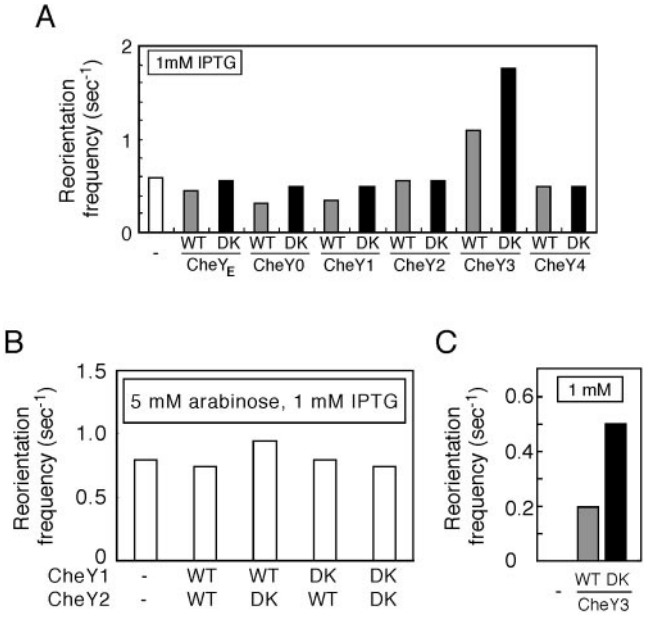

FIG. 4. Effects of CheY homologs on free swimming of *V. cholerae* cells in liquid medium. (A) Effects on chemotactic *V. cholerae* (O395N1) cells. (B) Effects of coexpression of CheY1 and CheY2 on chemotactic *V. cholerae* (O395N1) cells. (C) Effects of wild-type and mutant versions of CheY3 on nonchemotactic *V. cholerae* VcheA123 (Δ*cheA123*) cells. Overnight cultures of O395N1 or VcheA123 cells carrying plasmids were grown in the presence of 1 mM IPTG (A and C). CheY-coexpressing cells were grown in 1 mM IPTG and 0.5 mM arabinose (B). Cells were observed and analyzed as described in the legend to Fig. 3.

ented more frequently than those expressing the wild-type version (Fig. 4C). These results support the previous conclusion that only CheY3 of the five CheY homologs can induce CW rotation of the flagellar motor of *V. cholerae*.

Effects of CheY homologs on swimming behavior of *E. coli* cells. Unlike CheY3, both the wild-type and D-to-K mutant versions of $CheY_E$ could hardly promote the reorientation of *V. cholerae* cells. To test the exchangeability between *E. coli* and *V. cholerae*, the *V. cholerae* CheY homologs as well as $CheY_E$ were expressed in Che^+ *E. coli* cells (RP437) or cells lacking all Che proteins (ΔChe; HCB1262), and spreading of the resulting cells in tryptone semisolid agar was examined in the presence of 0.1 mM IPTG. The wild-type or mutant version of CheY3, but no version of the other CheY homologs, could induce CW rotation of the flagellar motor of *E. coli* (data not shown).

It has been reported that five of the six CheY homologs of *Rhodobacter sphaeroides* can rescue the chemotactic defect (tumbling) of an *E. coli cheZ* mutant strain (26, 29). We therefore examined the effects of the *V. cholerae* CheY homologs on spreading in semisolid agar of the *E. coli cheZ* mutant strain RP1616. Most of the CheY homologs, including CheY3, showed no detectable effect, but cells expressing the wild-type or D-to-K version of CheY4 formed a slightly larger ring with blurred edges indicative of an improved run/tumble bias. This increased spreading was not seen for cells expressing CheY4-D58N, in which the putative phosphorylatable aspartate residue was replaced (Fig. 5), although the mutant protein was expressed at a level similar to those of the wild-type and D-to-K versions (data not shown). Thus, the small effect of CheY4 on the *E. coli cheZ* strain is significant and may involve the phosphorylation of CheY4. These results suggest that CheY4 might affect flagellar rotation indirectly by receiving a phosphoryl group from CheY3 under some conditions.

Effects of substitutions at the interface between CheY and the flagellar motor protein FliM. Interestingly, $CheY_E$ only partially functioned in *V. cholerae*, whereas the *V. cholerae* CheY3 protein could act efficiently on the flagellar motor of *E. coli*. This asymmetry might be explained by the following amino acid substitutions in the interface between CheY and the flagellar switch protein FliM. The N-terminal sequence of FliM is highly conserved, but the *V. cholerae* FliM protein ($FliM_V$; MTDLLSQDEIDALLHGV) has Asp-8 in place of Ala-9 of the *E. coli* FliM protein ($FliM_E$; MGDSILSQAEIDALLNGD). Glu-117 of $CheY_E$ and Ala-9 of $FliM_E$ are in close proximity in the complex of $CheY_E$ and the N-terminal 16-residue peptide of $FliM_E$ (21). The former residue corresponds to Lys-120 of the CheY3 protein. Therefore, it was predicted that $FliM_V$ would disfavor $CheY_E$ due to an electric repulsion between Asp-8 and Glu-117, whereas an inverse combination of CheY3 and $FliM_E$ would be more tolerant.

To test this hypothesis of coevolution of the CheY-FliM pair, we introduced a Glu-117-to-Lys (E117K) mutation into $CheY_E$ and a Lys-120-to-Glu (K120E) mutation into CheY3. Immunoblotting revealed that the mutant proteins were expressed at levels similar to those of the wild-type versions (data not shown). *cheY3* knockout cells expressing CheY3-K120E reoriented less than cells expressing the wild-type version (Fig. 3B). On the other hand, cells expressing $CheY_E$-E117K spread (Table 2) and reoriented more frequently than those expressing the wild-type version (Fig. 3B). These results are consistent with the notion that residue Lys-120 of CheY3 is involved in switching of the flagellar motor of *V. cholerae*.

TABLE 4. Spreading in semisolid agar of *V. cholerae* Δ*cheA123* cells expressing each CheY homolog

CheY homolog	Mutation	Relative diameter[a]
None	NA[b]	0.53 ± 0.06
$CheY_E$	None	0.53 ± 0.02
	D13K	0.59 ± 0.05
CheY0	None	0.50 ± 0.02
	D11K	0.50 ± 0.06
CheY1	None	0.59 ± 0.05
	D10K	0.55 ± 0.02
CheY2	None	0.53 ± 0.02
	D10K	0.50 ± 0.06
CheY3	None	1.00 ± 0.05
	D16K	1.03 ± 0.08
CheY4	None	0.50 ± 0.02
	D15K	0.62 ± 0.08

[a] The relative diameters of swarm rings were measured as described in Table 1, except that the average diameter of the triplicate swarm rings of Δ*cheA123* cells expressing CheY3 was defined as 1.0, tryptone–semisolid 0.3% agar was supplemented with 0.1 mM IPTG, and the incubation time at 30°C was 24 h.

[b] NA, not applicable.

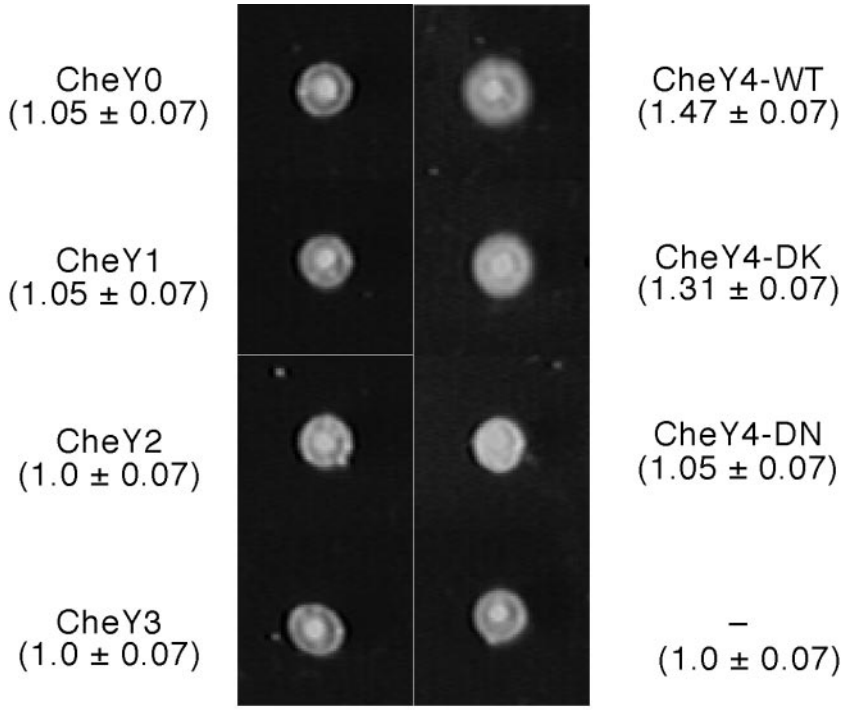

FIG. 5. Effects of CheY homologs on spreading of *E. coli cheZ* mutant cells in semisolid agar. Overnight cultures of RP1616 (*cheZ*) cells expressing each CheY-FLAG protein were spotted onto tryptone semisolid agar supplemented with 50 μg ml^{-1} ampicillin and 0.1 mM IPTG at 30°C for 12 h.

DISCUSSION

In this study, we examined the roles of the five different CheY homologs encoded in the genome of *V. cholerae* in swimming behaviors to determine which of the three *che* clusters are involved in chemotaxis. Deletion of the *cheY3* gene, but not that of the *cheY1*, *cheY2*, and *cheY4* genes, resulted in a defect in chemotaxis. This is consistent with a previous study looking at the requirement of the CheA homologs (12). In *R. sphaeroides*, all six CheY homologs, including those that are not involved in chemotaxis, bind to the motor switch protein FliM (11). Therefore, to assess whether each CheY homolog can control flagellar rotation directly, we examined the effect of CheY overexpression on swimming behavior rather than assaying the in vitro binding of CheY to FliM. Overproduction of the CheY3 protein, but not that of the other four CheY homologs, induced reorientation, indicating that only CheY3 directly controls flagellar rotation. Therefore, we concluded that only *che* cluster II is directly responsible for the (flagellum-mediated) chemotaxis of *V. cholerae* and that *che* clusters I and III as well as CheY0 might be involved in some physiological functions other than chemotaxis.

Among the CheA, CheB, and CheR homologs of *V. cholerae*, the most similar to those of *E. coli* are CheA3, CheB3, and CheR3, which are encoded in *che* cluster III (data not shown). In contrast, among the CheY homologs, the most similar to that of *E. coli* is CheY3, which is encoded in *che* cluster II. In particular, the FliM-interacting residues of $CheY_E$ are best conserved in CheY3, consistent with the finding that this is the only CheY homolog of *V. cholerae* that can regulate flagellar rotation.

We could not find phenotypes of *V. cholerae cheY* knockouts and overproducers, except for the *cheY3* mutant. Growth rates and cell morphologies of cells overproducing each CheY homolog were similar to those of wild-type cells (data not shown). Since CheY has no recognizable "output" domain (such as a DNA-binding domain), we suspect that the CheY homologs exert their functions by interacting with their target proteins. We failed to find a nonflagellar *V. cholerae* protein from the database with significant homology to the N-terminal amino acid sequence of the *V. cholerae* flagellar motor FliM protein that is deduced to serve as the target of CheY3. The *V. cholerae* CheY homologs other than CheY3 might interact with target sequences distinct from that of FliM.

It has been reported that duplication and deletion of *cheY4* in *V. cholerae* classical biotype strain 569B (serotype Inaba) increase and decrease spreading in LB semisolid agar as well as migration toward isolated intestinal mucus layers, respectively (3). This apparent discrepancy with our study might be accounted for by the difference in genetic background of the bacterial strains used or the polar effects of duplication and deletion of *cheY4*. Alternatively, *cheY4* might modulate chemotaxis indirectly. The effect of CheY4 on the *cheZ* mutant raises the intriguing possibility that CheY4 can affect chemotaxis though interaction with and phosphotransfer from CheY3. Another possibility is that the modulated chemotaxis is due to varied gene expression regulated by CheY4. Indeed, the duplication of *cheY4* increases the expression levels of *ctxAB* and *tcpA* (3). It has also been reported that motility and the expression of *ctxAB* are inversely regulated by bile (13). Therefore, it is possible that

CheY4 regulates the expression of a certain factor(s) that in turn affects chemotaxis under some condition(s). These possibilities should be tested experimentally.

Other genes in *che* clusters I and III might also contribute indirectly to the flagellum-mediated chemotaxis of *V. cholerae* by modulating the CheY3 function. For example, it is possible that CheY3 receives signals from noncognate CheA proteins under conditions that have not yet been identified. However, our data argue that flagellar rotation is modulated exclusively by CheY3. It has been reported that *che* genes in cluster II are involved in the expression of *ctxAB* and *toxT* genes (20). This suggests a link between chemotaxis itself and virulence. *V. cholerae* might be attracted by a factor in the intestine which functions as an inducer of expression of CT and Tcp genes, or *che* genes in cluster II might play a role in virulence gene expression. Chemotaxis might rather interfere with infection, although motility is required, since nonchemotactic mutants with counterclockwise biases of flagellar rotation outcompete the wild-type strain during mouse infections (6, 7). On the other hand, the MCP-like proteins TcpI and AcfB are also implicated in virulence (9, 15). Their roles in chemotaxis might be indirect unless they regulate the activity of CheA2, which seems unlikely from sequence comparisons (data not shown). Clearly, comprehensive genetic and biochemical analyses of this complex system are needed to clarify these issues.

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