INFECTION AND IMMUNITY, July 1994, p. 2669–2678
0019-9567/94/$04.00+0
Copyright © 1994, American Society for Microbiology
Vol. 62, No. 7

The *Vibrio cholerae* Toxin-Coregulated-Pilus Gene *tcpI* Encodes a Homolog of Methyl-Accepting Chemotaxis Proteins

CECIL W. HARKEY, KEITH D. EVERISS, AND KENNETH M. PETERSON*

Department of Microbiology and Immunology, Louisiana State University Medical Center, Shreveport, Louisiana 71130

Received 27 January 1994/Returned for modification 15 March 1994/Accepted 6 April 1994

Virulence gene activation in *Vibrio cholerae* is under the control of the ToxR-ToxT regulatory cascade. The ToxR regulon consists of genes required for toxin-coregulated-pilus (TCP) biogenesis, accessory colonization factor genes, cholera toxin genes, and ToxR-activated genes (*tag*) of unknown function. The *tagB* gene was isolated by using a *tagB*::Tn*phoA* fusion junction to probe a *V. cholerae* O395 bacteriophage lambda library. Nucleotide sequence analysis revealed that *tagB* is identical to *tcpI*, a gene which encodes a protein that negatively regulates the synthesis of the major pilin subunit of TCP (TcpA). Our results show that the *tcpI* gene encodes a 620-amino-acid protein that shares extensive sequence similarity with the highly conserved signaling domain in methyl-accepting chemotaxis proteins. Expression of *tcpI* in *Escherichia coli* results in the synthesis of a 71-kDa polypeptide that becomes localized to the inner membrane. Similarly, TcpI-PhoA alkaline phosphatase activity is enriched in *V. cholerae* inner membrane preparations. Colonies of *V. cholerae tcpI*::Tn*phoA* mutant cells display increased swarming on solid media when compared with those of the parental *V. cholerae* O395. Taken together, these observations suggest that TcpI may play a dual role in promoting vibrio colonization of the small bowel. In response to the appropriate environmental signal(s), TcpI permits maximum expression of *tcpA* while simultaneously reducing vibrio chemotaxis-directed motility. We believe coordinate regulation of colonization and motility determinants, in such a fashion, facilitates efficient *V. cholerae* microcolony formation.

Vibrio cholerae is the etiologic agent of the diarrheal disease Asiatic cholera. The manifestations of this infection are a result of the bacterium's ability to colonize the surface epithelium of the small bowel and to produce numerous exoproteins, including the potent enterotoxin encoded by the *ctxAB* operon (4, 10, 13, 14, 16, 17, 19, 32, 54). The expression of at least 17 *V. cholerae* determinants (ToxR regulon) involved in these processes is under the control of the ToxR-ToxT regulatory cascade (10–12, 25, 32, 34–36, 45). The ToxR regulon was defined primarily through screening a large collection of random *V. cholerae* Tn*phoA* fusion strains for ToxR-regulated expression of alkaline phosphatase activity. These studies indicated that ToxR regulon genes fall into four general categories: genes involved in the biogenesis of the toxin-coregulated pilus (TCP), cholera toxin genes, accessory colonization factor genes, and ToxR-activated genes (TAG) that do not affect *V. cholerae* colonization, pilus biogenesis, or toxin synthesis and secretion (10, 43, 45).

Induction of the ToxR regulon in vitro can be affected by incubation temperature, pH, osmolarity, oxygen tension, and the amino acid composition of the medium via a signal transduction pathway that is not understood (10, 33). Although the intraintestinal factors that regulate the expression of the ToxR regulon have not been elucidated, it is clear that environmental activation of the ToxR regulon is dependent upon the hierarchical expression of at least two activators, ToxR and ToxT (11, 12, 25). Recent reports have demonstrated that *V. cholerae* virulence gene expression requires the ToxR-dependent activation of *toxT* and then ToxT activation of the *tcp*, *acf*, and *tag* genes (11, 12, 25). It is not known whether ToxR directly or indirectly activates *toxT* expression. The regulatory cascade model that is emerging suggests that ToxR controls the synthesis of a number of ToxR regulon determinants by activating the expression of *toxT* (12).

V. cholerae O395 synthesizes a type 4 pilus that was designated TCP since the growth conditions that lead to maximum pilus production were observed to be also optimal for toxin synthesis. Both pilus production and toxin production are regulated by *toxR* (53). The *V. cholerae tcpA* gene encodes the major pilin subunit that is assembled into a cell-surface colonization determinant via the action of at least seven accessory proteins (26, 27, 37, 44, 45, 49, 51, 53). Challenge studies utilizing a *tcpA* deletion mutant of *V. cholerae* O395 demonstrated that *V. cholerae* requires TCP for enterocolonization of human volunteers (24). Characterization of *tcpA* and other genes required for pilus biogenesis and function has begun to reveal the key steps in the TCP biogenesis pathway. An early step in this process involves the proteolytic cleavage of the hydrophobic TcpA leader peptide by the TcpJ protein (27). A *tcpG*-encoded thiol:disulfide isomerase is required for the functional maturation of the TCP (44). Tn*phoA* insertion mutations have identified several other genes involved in TCP biogenesis (26, 45, 51). Most of these mutations result in a TCP$^-$ phenotype, i.e., they confer loss of autoagglutination under the appropriate culture conditions, loss of fucose-resistant hemagglutination, and a defect in colonization of infant mice (26, 27, 44, 45, 49, 51, 53).

Transcriptional control of the genes involved in pilus biogenesis is coordinated by ToxR (34–36). Recent studies have shown that positive regulation of *tcpA* transcription requires the ToxT protein as part of the ToxR-ToxT regulatory cascade (12, 25). Directed mutagenesis of sequences immediately upstream of the *tcpA* structural gene has identified two ToxR-regulated genes that affect TcpA synthesis, *tcpH* and *tcpI*. Inactivation of *tcpH* results in decreased pilin synthesis,

* Corresponding author. Mailing address: Department of Microbiology and Immunology, LSU Medical Center, 1501 Kings Highway, Shreveport, LA 71130. Phone: (318) 674-5753. Fax: (318) 674-5764.

TABLE 1. Bacterial strains, plasmids, and bacteriophage used in this study

Strain, plasmid, or phage	Relevant genotype or characteristics[a]	Source or reference
V. cholerae		
O395 Sm	Str^r	53
KP5.51	O395 *tcpI*::Tn*phoA* Str^r Kan^r	45
KP7.74	O395 *tcpI*::Tn*phoA* Str^r Kan^r	45
KP1.17	O395 *toxR12* Str^r Amp^r	Laboratory collection
E. coli		
ER1451	*mcrB mcrA* F'(del*lac*ZM15)	New England Biolabs
DH11S	*mcrC* del(*mrr hsdRMS mcrBC*) del(*lac-proAB*) del*recA* F'(del*lac*ZM15)	Bethesda Research Labs
KW251	*supE44 galK2 galT22 metB1 hsdR2 mcrB1 mcrA* (*argA81*::Tn*10*) *recD1014*, F$^-$ Tet^r	Promega
Plasmids		
pBluescriptII KS$^+$	LacZ' Amp^r	
pUC18	LacZ' Amp^r	
pFTCPI	pUC18 containing 10.6-kb *Bam*HI-*Xba*I fragment from KP5.51 (*tcpI*::Tn*phoA*') Kan^r Amp^r	This study
pTCPI-1	pBluescriptII KS$^+$ containing 4.0-kb *Sca*I fragment from λGem11.551(*orfC tcpI*) Amp^r	This study
pTCPI-2	pBluescriptII KS$^+$ containing 1.0-kb *Sac*I-*Sca*I fragment from λGem11.551 ('*tcpI*) Amp^r	This study
pTCPI-3	pBluescriptII KS$^+$ containing 3.5-kb *Cla*I-*Sca*I fragment from pTCPI-2 (*orfC tcpI*') Amp^r	This study
pTCPI-4	pBluescriptII KS$^+$ containing 360-bp *Eco*RV-*Cla*I fragment from pTCPI-3 ('*tcpI*') Amp^r	This study
pTCPI-5	pBluescriptII KS$^+$ containing 1-kb *Eco*RV fragment from pTCPI-1 ('*tcpI*') Amp^r	This study
pTCPI-6	pBluescriptII KS$^+$ containing 1-kb *Nsi*I-*Ssp*I fragment from pTCPI-1 ('*orfC tcpI*') Amp^r	This study
pTCPI-7	pBluescriptII KS$^+$ containing 600-bp *Eco*RV fragment from pTCPI-1 (*orfC tcpI*') Amp^r	This study
pTCPI-8	pBluescriptII KS$^+$ containing 1.8-kb *Dra*I fragment from pTCPI-1 (*tcpI*) Amp^r	This study
pTCPI-9	pBluescriptII KS$^+$ containing 1.0-kb *Ssp*I-*Xba*I fragment from pTCPI-1 ('*tcpI*') Amp^r	This study
pTCPI-10	pBluescriptII KS$^+$ containing 3.5-kb *Cla*I fragment from pTCPI-1 ('*tcpI*) Amp^r	This study
pTCPI-11	pBluescriptII KS$^+$ containing 1.6-kb *Nsi*I-*Pst*I fragment from pTCPI-1 (*orfC*') Amp^r	This study
Bacteriophage λ		
λGem11	Cloning vector	Promega
λGem11.551	λGem11 containing 14-kb partial *Sau*3A fragment from O395 Sm	This study

[a] Abbreviations: Amp^r, ampicillin resistance; Kan^r, kanamycin resistance; Str^r, streptomycin resistance; Tet^r, tetracycline resistance.

whereas inactivation of *tcpI* leads to increased synthesis of TcpA (51). Presently, it is not known at what level *tcpI* and *tcpH* affect TcpA synthesis. It has been suggested that regulators such as TcpI that act downstream of ToxR and ToxT may function to fine-tune the expression of the TCP virulence determinant throughout the *V. cholerae* pathogenic cycle (12).

The findings presented in this report show that the previously described *tagB*::Tn*phoA* insertion lies within *tcpI*, a gene that encodes a negative regulator of *tcpA*, and suggest that TcpI belongs to a family of proteins involved in environmental sensing and signal transduction. TcpI may provide *V. cholerae* a mechanism for sensing the environment such that, at the appropriate time and/or place, maximum expression of the TCP colonization determinant occurs.

MATERIALS AND METHODS

Bacterial strains, plasmids, media, and reagents. The *Escherichia coli* and *V. cholerae* strains and plasmids used in this study are listed in Table 1. *V. cholerae* and *E. coli* strains were maintained at −70°C in Luria-Bertani (LB) medium containing 25% (vol/vol) glycerol. *E. coli* strains were cultured in LB medium at 37°C. *V. cholerae* strains were cultured in LB medium at pH 6.5 at 30°C or in LB medium at pH 8.4 at 37°C as described previously (45). Ampicillin (100 μg/ml), streptomycin (100 μg/ml), kanamycin (45 μg/ml), gentamicin (30 μg/ml), and rifampin (200 μg/ml) were used where appropriate. Unless otherwise indicated, all chemical reagents were obtained from Sigma Chemical Co. (St. Louis, Mo.). Isotopes for radiolabeling protein, [^{35}S]methionine and -cysteine, were purchased from New England Nuclear (Boston, Mass.). Radionucleotides for radiolabeling nucleic acid, [α-^{32}P]dATP or [α-^{35}S]dATP, were obtained from Amersham (Arlington Heights, Ill.).

Genomic library construction. Genomic DNA from *V. cholerae* O395 was subjected to partial *Sau*3A digestion as described by Maniatis et al. (30). Endonuclease-treated DNA was fractionated by centrifugation on a linear 10 to 40% sucrose gradient. DNA fragments of 10 to 20 kbp were pooled and dialyzed against Tris-EDTA buffer (pH 8.0) and then subjected to phenol extraction and ethanol precipitation. *Bam*HI-digested λGem-11 DNA (Promega Corp., Madison, Wis.) was ligated to the sized *V. cholerae* DNA fragments, packaged in vitro by using a Packagene lambda DNA packaging system (Promega) to produce viable phages, and used to infect strain KW251 that had been grown in the presence of 0.4% maltose and 1 mM $MgSO_4$.

Cloning the *tagB*::Tn*phoA* fusion and the intact *tcpI* gene. Chromosomal DNA isolated from *V. cholerae* KP5.51 (*tagB*::Tn*phoA*) was digested with *Bam*HI and *Xba*I, ligated into similarly digested pBluescriptII KS$^+$ (Stratagene, La Jolla, Calif.), and transformed into *E. coli* DH11S (Bethesda Research Laboratories, Bethesda, Md.). Transformants were selected on LB agar containing kanamycin and ampicillin. A single recombinant plasmid (pFTCPI) was chosen for further study. The cloned *tagB*::Tn*phoA* fusion junction from pFTCPI was used to probe a *V. cholerae* O395 library constructed in bacteriophage λGem11. Recombinant phage plaque material was immobilized on nitrocellulose discs and screened by in situ hybridization (30).

Subcloning and DNA sequence analysis. Restriction endonuclease fragments containing *tcpI* or the *tagB*::Tn*phoA* fusion

junction were inserted into pBluescriptII KS$^+$ (Table 1). Plasmids carrying the appropriate inserts, determined by restriction enzyme analysis, were subjected to double-stranded DNA sequencing. Plasmids were sequenced by using synthesized oligonucleotide primers (47) and Sequenase version 2.0 (U.S. Biochemical Corp., Cleveland, Ohio) as described in the manufacturer's protocol. Sequencing products were resolved on 6.0% polyacrylamide gels that were fixed, dried, and exposed to Kodak X-Omat film overnight at room temperature. The MacVector 4.1 software package (IBI, New Haven, Conn.) was used to process and analyze nucleic acid and protein sequence data.

Synthesis of TcpI in *E. coli*. T7-directed expression of *tcpI* was performed as outlined previously (50) except that T7 induction occurred by infection with M13/T7 (Invitrogen, San Diego, Calif.). *E. coli* cells transformed with pTCPI-1 were grown in LB broth, containing ampicillin, to the mid-log stage, harvested by centrifugation, washed, and resuspended in a defined medium containing all common amino acids except methionine and cysteine. The cells were then grown for 1 to 2 h and infected with M13/T7 at a multiplicity of infection of approximately 10. Isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 1 mM to induce expression of the T7 RNA polymerase gene on M13/T7. After 30 min, rifampin was added and incubation was continued for 40 min prior to a 5-min pulse of 10 μCi of [^{35}S]methionine-cysteine. For signal peptidase I inhibition studies, ethanol or sodium azide was added to the cells 15 min into the rifampin block to a final concentration of 9% or 4 mM, respectively (27, 39). Cells were harvested by centrifugation and resuspended in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) solubilization buffer (60 mM Tris-HCl [pH 6.8], 1% mercaptoethanol, 8 M urea, 10% glycerol, 3% SDS, and 0.01% bromophenol blue). Solubilized cell extracts were boiled for 3 min prior to SDS-PAGE. Gels were treated with Enlightning (New England Nuclear Research Products, Boston, Mass.), dried, and exposed to Kodak X-Omat film.

Subcellular localization of TcpI. *E. coli* and *V. cholerae* cells were fractionated by the method of Kelley and Parker (28) with some modifications. Frozen cell pellets were thawed and resuspended in either 1/10 volume (*V. cholerae*) or 1/20 volume (*E. coli*) of 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; buffer pH 7.4) containing 18% (wt/wt) sucrose. DNase (3 μg/ml) was added to the suspension, and the mixture was incubated for 30 min at 4°C. The cell suspension was then passed through a French pressure cell at 20,000 lb/in^2. Unbroken cells were removed by centrifugation at 5,000 × *g* for 20 min. The remaining cell lysate was diluted with an equal volume of HEPES buffer and layered onto a sucrose step gradient containing 1 ml of 55% (wt/wt) sucrose and 1 ml of 14% (wt/wt) sucrose in HEPES buffer. The gradients were then centrifuged for 2 h at 33,000 rpm in an SW41 rotor. The top 3 ml from each gradient was collected and incubated at 23°C for 30 min prior to centrifugation at 200,000 × *g* for 1 h to yield the soluble fraction (cytoplasm and periplasm). The membrane fraction was isolated, washed in 3 volumes of HEPES buffer, and collected via centrifugation. Inner and outer membrane fractions were separated via isopycnic sucrose density gradient fractionation (48). Briefly, samples (approximately 5 mg of protein) were layered onto a sucrose gradient (1 ml of 55%, 2.5 ml of 50%, 2.5 ml of 45%, 2.5 ml of 40%, and 2.5 ml of 35% [wt/wt] sucrose in HEPES buffer) and centrifuged for 24 to 30 h in an SW41 rotor at 33,000 rpm. Fractions were isolated dropwise from the bottoms of the tubes. The membrane-containing fractions were identified by measuring the protein concentrations of each fraction with the bicinchoninic acid reagent as recommended by Pierce Chemical Co. (Rockford, Ill.). It was determined that the outer membrane fractions were contaminated less than 10% by inner membranes on the basis of succinate dehydrogenase activity (38). Inner and outer membrane fractions were also isolated by *N*-lauroyl sarcosine extraction of total membrane fractions (15). Alkaline phosphatase activity was determined by adding 5 μg of protein in 20 μl of HEPES buffer (pH 7.4). Each sample then received 160 μl of 0.05% *p*-nitrophenyl phosphate in 1 M Tris-Cl (pH 8.0) (45). Alkaline phosphatase activity was calculated as follows: $[(A_{420} - A_{570})/20] \times 100$.

Swarm plate assay. Five microliters of cells from an overnight culture of *V. cholerae* that was grown under conditions that repress TAG expression (LB medium at pH 8.4) was inoculated into LB medium at pH 6.5 (conditions that promote TAG expression) containing 0.3% agar with the appropriate antibiotics and cultured at 30°C for 24 h.

Nucleotide sequence accession number. The *tcpI* sequence has been deposited in the GenBank data base under accession number L25659.

RESULTS

Cloning of the *tagB*::Tn*phoA* fusion junction. The *tagB* gene was initially identified by the Tn*phoA* insertion in *V. cholerae* KP5.51 (Table 1) which lies within *tagB* and creates a gene fusion between *tagB* and *phoA*. This *tagB*::Tn*phoA* fusion junction was inserted into *Bam*HI-*Xba*I-digested pUC18 by selecting for transposon-encoded kanamycin resistance. The *tagB*::*phoA* fusion junction was then sequenced with a *phoA* primer (52) to determine the *tagB* reading frame. The resulting sequencing reactions yielded a 150-nucleotide open reading frame that was identical to a partial sequence of the *V. cholerae tcpI* gene present in the National Center for Biotechnology Information integrated data base (GenBank). Henceforth, we will refer to the gene defined by the Tn*phoA* insertion in *V. cholerae* KP5.51 as *tcpI*.

Cloning of the gene encoding TcpI (*tcpI*). To isolate the intact *tcpI* gene, a *V. cholerae* O395 genomic library was constructed in bacteriophage λGem-11. Recombinant bacteriophages which carried *tcpI* sequences were identified by in situ hybridization of bacteriophage λ plaques by using a 3.5-kbp *Cla*I fragment from pFTCPI as a *tcpI*-specific probe. A single recombinant bacteriophage λGem11 clone, designated λGem11-551, was isolated and characterized by restriction analysis (Fig. 1). A 4.0-kbp *Sca*I fragment from λGem11-551 was inserted into pBluescriptII KS$^+$ and designated pTCPI-1 (Fig. 1).

DNA sequence of *tcpI*. To characterize and define *tcpI*, various restriction fragments of pTCPI-1 DNA were inserted into pBluescriptII KS$^+$ (Table 1) and sequenced by the dideoxynucleotide method (47). Both strands of the 4.0-kbp *Sca*I fragment of pTCPI-1 were sequenced. The *tcpI* nucleotide and deduced protein sequences are shown in Fig. 2. Analysis of the *tcpI* structural gene revealed an 1,860-nucleotide open reading frame that could encode a 69-kDa protein with an isoelectric point of 4.72. Three additional open reading frames, all transcribed in the opposite direction of *tcpI*, were located within the cloned *Sca*I fragment (Fig. 1). Open reading frame 1 is predicted to encode a 164-amino-acid protein that exhibits a high degree of similarity to a 20-kDa protein of unknown function in the *Streptococcus sanguis* type 12 fimbria gene cluster (data not shown). Open reading frame 2 is predicted to encode a 326-amino-acid polypeptide with no significant similarity to any proteins in GenBank. A third, partial open reading frame encoding 93 amino acids was also

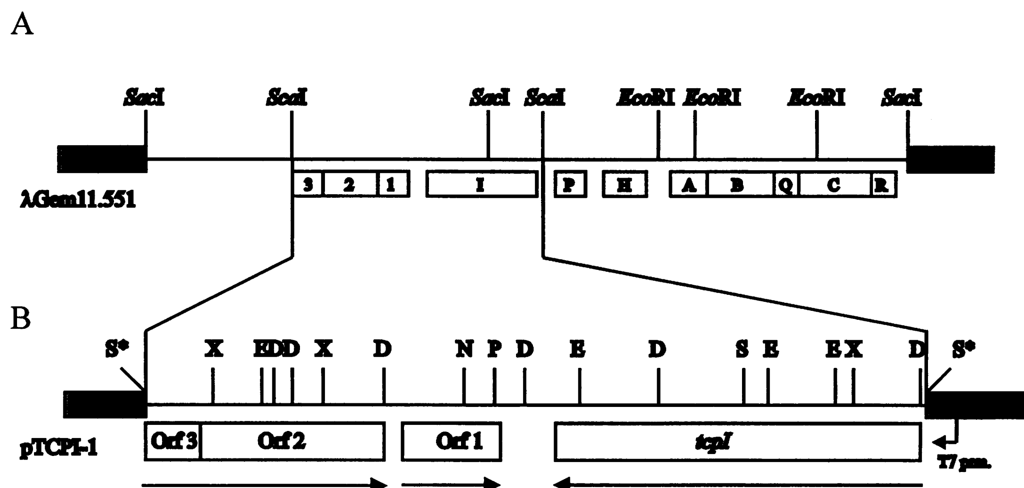

FIG. 1. (A) Schematic representation of the *tcp* locus present in the λGem11.551 recombinant bacteriophage. Thin lines represent *V. cholerae* sequences. Restriction sites are noted above the line, and the hatched boxes represent bacteriophage λ sequences. Numerals 1, 2, and 3 denote open reading frames identified by sequence analysis. The letters I, P, H, A, B, Q, C, and R refer to previously identified *tcp* genes. (B) Schematic illustration of pTCPI-1 containing the 4.0-kb *Sca*I fragment from λGem11.551. The thin line represents *V. cholerae* sequences, and the hatched boxes refer to vector sequences. Restriction sites are abbreviated as follows: D, *Dra*I; E, *Eco*RI; N, *Nsi*I; P, *Pvu*I; S, *Sac*I; X, *Xba*I. Asterisks denote *Sca*I restriction sites that have been disrupted. Orf1, Orf2, and Orf3 denote open reading frames. The direction of the T7 polymerase promoter in pBluescriptII KS$^+$ is indicated by an arrow labeled T7 prm. The other arrows indicate the direction of transcription.

detected but failed to show significant relatedness to any proteins in the GenBank data base.

Comparison of TcpI with the GenBank protein data base revealed a high degree of similarity between TcpI and a family of integral inner membrane proteins involved in regulating bacterial chemotaxis. This family includes the methyl-accepting chemotaxis proteins (MCPs) from a wide variety of procaryotic genera which serve as environmental sensory molecules (5, 6, 7, 20–22, 31, 40, 41). The greatest degree of amino acid sequence similarity between TcpI and MCPs is present in a highly conserved domain (HCD) present in the signaling domain of the Tsr MCP (1). This region of the MCP is believed to interact directly with CheW, a modulator of the signaling process (29). A similar domain is found in other bacterial proteins that are not associated with chemotaxis. These include HlyB of *V. cholerae*, a protein implicated in the secretion of hemolysin (HlyA) (3), and PilJ, a *Pseudomonas aeruginosa* protein required for the production of type 4 fimbriae (9). The amino acid sequence similarity is confined to the carboxyl half of the proteins as illustrated in the aligned sequences (Fig. 3). TcpI exhibits 23, 18, and 17% amino acid identities with HlyB, PilJ, and Tsr, respectively, and overall similarities of 41, 40, and 38% to these same proteins. Also conserved in TcpI are potential sites of methylation that are located in the K1 and R1 domains of Tsr (46). We do not yet know if TcpI is methylated.

Structural features of TcpI. Located at the amino terminus of TcpI is a potential procaryotic signal peptide with possible peptidase cleavage sites located between residues 19 and 20 or 21 and 22 (55). The localization of the TcpI-PhoA fusion protein to *V. cholerae* inner membranes (Fig. 4) indicates that cleavage of the TcpI signal peptide does not occur. In the absence of this cleavage event, the hydrophobic amino terminus serves as a transmembrane domain that anchors TcpI to the inner membrane. Computer algorithms determined that TcpI possesses another stretch of hydrophobic amino acids that is predicted to represent a membrane-spanning alpha helix. Similar hydrophobic domains are spatially conserved in Tsr, PilJ, and HlyB (Fig. 5). The overall hydrophilicity pattern of the predicted TcpI polypeptide is similar to that of Tsr, suggesting that TcpI may have a membrane topology that is identical to that of the MCPs. Limited Tn*phoA* and Tn*lacZ* mutagenesis of *tcpI* supports this prediction. Enzymatically active TcpI-PhoA fusions only occurred when PhoA was fused to the putative TcpI periplasmic domain. Conversely, enzymatically active TcpI-LacZ hybrids were produced only when LacZ was fused to the predicted cytoplasmic domain of TcpI (data not shown).

Heterologous expression of the *V. cholerae tcpI* gene in *E. coli*. The T7 promoter provided by the pBluescriptII KS$^+$ plasmid derivative pTCPI-1 (Fig. 1) was used to examine TcpI synthesis in *E. coli*. ER1451 cells transformed with pBluescriptII KS$^+$ served as a control. Synthesis of TcpI was induced by infection of pTCPI-1-transformed *E. coli* cells with recombinant M13 phage M13/T7 and the addition of IPTG. T7-directed expression of *tcpI* resulted in the synthesis of a unique ^{35}S-labeled protein estimated to be 71 kDa in size (Fig. 6). The molecular weight of the TcpI protein synthesized in *E. coli*, determined by SDS-PAGE, was in good agreement with the predicted molecular mass of the TcpI protein. Pulse chase (data not shown) and signal peptidase I inactivation experiments (Fig. 6, lanes 5 and 7) failed to identify a higher-molecular-weight form of TcpI, suggesting that TcpI is not synthesized as a larger precursor protein. In control experiments, ethanol and sodium azide inhibited the processing of AcfC and AcfA, two vibrio proteins with characteristic signal peptidase I cleavage sites (data not shown).

Localization of TcpI in *E. coli* and *V. cholerae* cells. As noted earlier, TcpI possesses significant amino acid similarity to the signaling domain of MCPs, a class of integral inner membrane proteins that possess well-characterized cytoplasmic and periplasmic domains (21, 22, 40, 41). Fractionation of *E. coli* cells synthesizing TcpI and fractionation of *V. cholerae* cells synthesizing a TcpI-PhoA hybrid protein (alkaline phosphatase fused to the N-terminal amino acids of TcpI) were performed to determine the subcellular location of TcpI. As shown in Fig. 7, T7-directed expression of the *tcpI* structural

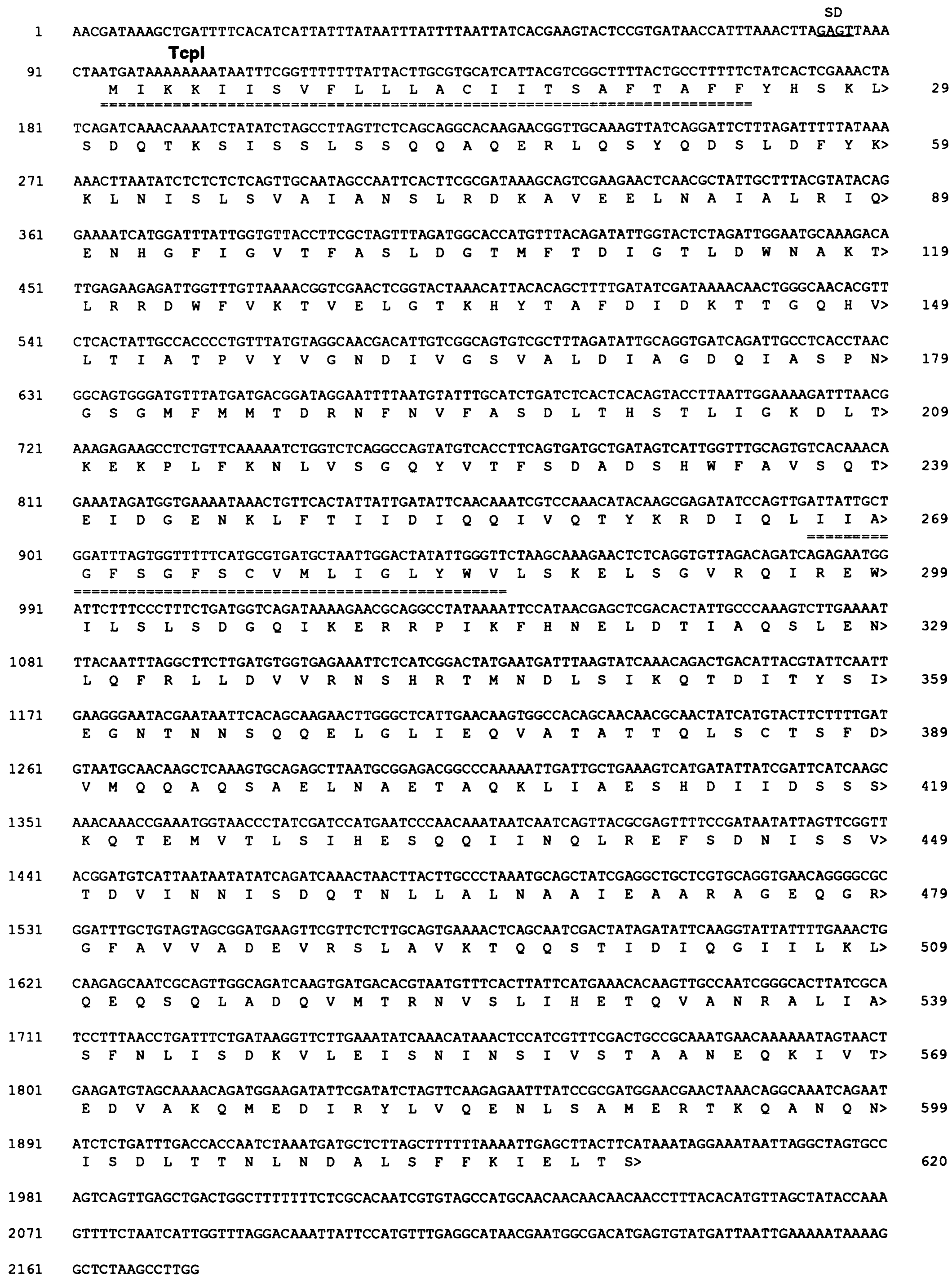

FIG. 2. Nucleotide sequence of the *V. cholerae tcpI* gene. The numbers on the left denote nucleotide positions, with the numbering starting at the *Dra*I site and ending at the *Sca*I site (position 2174), and the numbers on the right refer to amino acid positions within TcpI. The putative Shine-Dalgarno element is underlined and indicated by SD. TcpI refers to the start of the TcpI polypeptide. The double underlining denotes hydrophobic amino acids arranged in alpha helixes that are predicted to represent membrane-spanning regions within TcpI.

gene in *E. coli* yielded a 71-kDa protein that localized to the total membrane fraction and the sarcosyl-soluble (inner membrane) fraction. Fractionation of *V. cholerae* cells containing a *tcpI*::Tn*phoA* insertion showed that the alkaline phosphatase activity produced by *V. cholerae* KP5.51 localized to the inner membrane (Fig. 4). Western blot (immunoblot) analysis of *V. cholerae* KP5.51 inner membrane preparations indicated that the TcpI-PhoA protein was of the predicted size on the basis of the location of the Tn*phoA* insertion in *tcpI* (data not shown).

TcpI affects *V. cholerae* swarm plate activity. *V. cholerae* cells

```
TcpI     MIKK--------------IISVFLLLACIITSAFTAFFYHSKLSDQTK--------------SISSLSSQQAQER   47
HlyB     MIIN--------------KFSLKWMLAIAVAIPAIRLLFVA-----FT--------------SLNTMSVMQAQSN   42
PilJ     MKKINAGNLFAGMRSSSVIAGLFIVLIVSIVLLFANFAYLNTQSNHDKQYIGHAGELRVLSQRIAKNATEAAAGK   75
Tsr      M-----------LKRIKIVTSLLLVLAVFGLLQLTS------------------GGL------------------   28
         *                   ..  .*

TcpI     LQSY----QDSLDFYKKLNISLSVAIANSLRDKAVEELNAIALRIQENHGFIGVTFASLDGTMFTDIGTLDWNAK  118
HlyB     --------------------SLYANTAAPMRAMAEATSRIPRMRV-------GIDMMLLQETALKDAKGV-----   85
PilJ     GEAFKLLKDARNDFEKRWNILVNGDESTSLPPSPEAVKPQMDVVQQDWDGLRKNADSILASE--QTVLSLHQVAS  148
Tsr      --FFNALKNDKENF------------------------TVLQTIRQQQSTLNGSWVALLQTR------------N   65
                                                                   *..

TcpI     TLRRDWFVKTVELGTKHYTAFDIDKTTGQHVLTIATPVYVGNDIV---GSVALDIAGDQIASP---------NGS  181
HlyB     -LKR------------------VEEARTEDIPEMRQRMQVAVDSQ---VNPELKEQARKLQAR---------FEQ  129
PilJ     TLAET--IPQLQVEYEEVVDILLENGAPADQVAVAQRQSLLAERILGSVNKVLAGDENSVQAADSFGRDASLFGR  221
Tsr      TLNRA------------GIRYMMDQNNIGSGSTVAE--------LMESASISLKQAEKNWADYEALPRDPRQ-ST  119
          *                                                                       .

TcpI     GMFMMTDRNFNVFASDLTHSTLIGKDLTKEKPLFK---------NLVSGQYVTFSDADSHWFAVSQTEIDGENKL  247
Hlyb     MVREELEPMLQAFANN---------DMTTAQNIYR---------DKYAPTYGEMRKQANQ---ILDTLLQQADR-  182
PilJ     VLKGMQEGNAAMSISKVTNAEAVDR-LNEIAELFEFVSGSVDEILETSPDLFQVREAANNIFSVSQTLLDKASQL  295
Tsr      AAAAEIKRNYDIYHNALAELIQL---LGAGKSTSSLISRPRDIRNGFEKQYVAY--------------MEQNDRL  167
               .   .   .           ..                   .                    .. ...

TcpI     FTIIDIQQIVQTYK--RDIQLIIAGFS----GFSCVMLIGLYWVLSKELSG-VRQIREWI-LSLSDG-QIKERRP  313
HlyB     ----QNHASVESFEAGRTKQMVIIA-A----GLIISFITSL--VIITNLRSRVAYLKDRM-SSAAANLSLRTRLE  245
PilJ     ------ADGFENLAGGRSINL-FAGYALGALALASIILIGL--VMVRETNRRLAETAEKNDRNQAAILRLLDEIA  361
Tsr      HDI-AVSDNNASYSQAM---WILVGVMIVVLA----VIFAVWFGIKASLVAPMNRLIDSIRHIAGGDL--VKPIE  242
                .  ..    .     .  .      .     . ..   .  .    .    .      ..     .

TcpI     IKFHNELDTIAQSLENLQFRLLDVVRNSHRTMNDL----SIKQTDITYSIEGNTNN-------SQQELGLIEQVA  377
HlyB     LDGNDELCDIGKSFNAFIDKVHHSIEEVAENSKEL----ATMASSVSQRAHMTQSN-------CASQRDRTVQVA  309
PilJ     DLADGDLTVAATVTEDFTGAIADSINYSIDQLRELVETINQTAVQVAAAAQETQSTAMHLAEASEHQAQEIAGAS  436
Tsr      VDGSNEMGQLAESLRHMQGELMRTV----GDVRNGANAIYSGASEIATGNND-------LSSRTEQQAASLEETA  306
             ...   .     .   .   .       ..        .  ..   .             . .  K1  ..

TcpI     TATTQLSCTSFDVMQQAQSAELNAETAQKLIAESHDIIDSSSKQTEMVTLSIHESQQIINQLREFSDNISSVTDV  452
HlyB     TAIHELGATVSEIASNAAMARDVANEATLHSGEGKKVVGEVQNRIQTLVNELDNATQVVSSLATQINGISSTLDT  384
PilJ     AAINEMAVSIDQVSANASESSAVAERSVAIANKGNEVVHNTITGMDNIREQIQDTSKRIKRLGESSQEIGDIVSL  511
Tsr      ASMEQLTATVKQNAENARQASHLALSASETAQRGGKVVDNVV--------------QTMRDISTSSQKIADIISV  367
         ..  ... .  .   .*  .   *  .      . ... .                . .. . .  .  *... .

TcpI     INNISDQTNLLALNAAIEAARAGEQGRGFAVVADEVRSLAVKTQQSTIDIQGIILKLQEQSQLADQVMTRNVSLI  527
HlyB     IRSISEQTNLLALNAAIEAARAGEQGRGFAVVADEVRTLASRSAASTEEIQQVINRLQTESTRAVEAMEKGRSQS  459
PilJ     INDIADQTNILALNAAIQASMAGDAGRGFAVVADEVQRLAERSSAATKQIEALVKTIQTDTNEAVISMEQTTSEV  586
Tsr      IDGIAFQTNILALNAAVEAARAGEQGRGFAVVAGEVRNLAQRSAQAAREIKSLI--------------EDSVGKV  428
         * .*. ***.******..*..**..********.**. ** .. ... .*. ..              . . .

TcpI     HETQVANRALIASFNLISDKVLEISNINSIVSTAANEQKIVTEDVAKQMEDIRYLVQENLSAMERTKQANQNISD  602
HlyB     DVVVEFSAKANQSLTEINSQIDQINDQNIQVATATEEQSTVVEDINRNVEDINQLTTETSHVADELSRASASLQR  534
PilJ     VRGARLAQDAGVALEEIEKVSKTLAALIQNISNAARQQASSAGHISNTMNVIQEITSQTSAGTTATARSIGNLAK  661
Tsr      DVGSTLVESAGETMAEIVSAVTRVTDIMGEIASASDEQSRGIDQVGLAVAEMDRVTQQNAALVEESAAAAAALEE  503
                     ... * .    ...    ...*. .*    ....  .. .  .. .. R1 .    . . .

TcpI     LTTNLNDALSFFKIEL------------T---S   620
HlyB     LSSQLDKLVGSFEL-------------------   548
PilJ     MASEMRNSVSGFKLPE------------GVEQA   682
Tsr      QASRLTEAVAVFRIQQQQRETSAVVKTVTPAAP   536
          .. . . .. * .
```

FIG. 3. Multiple sequence alignment of *V. cholerae* TcpI and HlyB, *P. aeruginosa* PilJ, and *E. coli* Tsr. The numbers on the right indicated amino acid positions within the four proteins. Dash marks (−) denote gaps inserted into the sequences to improve the alignments. Residues conserved in all four sequences are indicated by asterisks (*). Highly conserved residues present in all four sequences are indicated by periods (.). K1 and R1 refer to the methylation domains of Tsr and are underlined. The highly conserved domain of Tsr is underlined and further denoted by bold italicized letters.

grown under conditions in which the ToxR regulon is maximally expressed display reduced motility in a swarm plate assay (18). The finding that *E. coli* cells altered in their ability to react to chemical stimuli exhibit diminished swarm plate activity suggests that ToxR may somehow regulate the chemotactic response of *V. cholerae*. These observations, and the presence of the highly conserved signaling domain of MCPs within TcpI, prompted us to examine *V. cholerae* KP5.51 and KP7.74 (*tcpI*::Tn*phoA*) for altered motility in a swarm plate assay. The swarm plate phenotype of four *V. cholerae* strains is shown in Fig. 8. *V. cholerae* KP1.17 carrying an internal deletion in the *toxR* structural gene and O395 derivatives with a *tcpI*::Tn*phoA* insertion mutation display increased swarm plate activity when compared with that of the parental O395 cells. These results suggest that the effect of *toxR* and *tcpI* mutations is an increase in swarming on the part of the mutants compared with the wild-type cells. At present, we do not know whether the increased swarming of *toxR* and *tcpI* mutant vibrio cells in semisolid medium is the result of hypermotility or an alteration in chemotaxis-directed motility.

DISCUSSION

The *tagB*::Tn*phoA* insertion was cloned from *V. cholerae* KP5.51, a fully virulent, TCP-expressing, infant mouse colonization-competent O395 derivative (45). The *tagB*::*phoA* fusion junction was used to determine the *tagB* gene sequence and as a probe to isolate the intact gene. Computer-aided searches of the National Center for Biotechnology Information integrated data base revealed that *tagB* sequences were identical to those

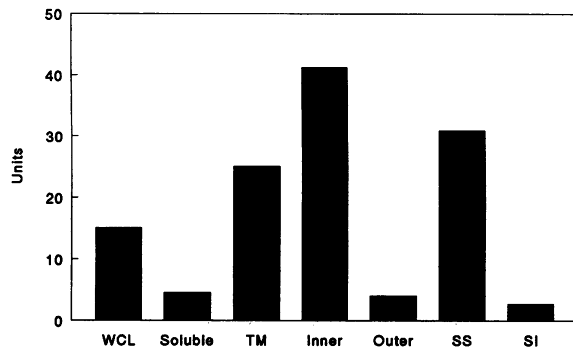

FIG. 4. Subcellular localization of the TcpI-PhoA hybrid protein activity in *V. cholerae* KP5.51. Abbreviations: WCL, whole-cell lysate; soluble, cytoplasm and periplasm; TM, total membrane fraction; inner, inner membranes; outer, outer membranes; SS, sarcosyl-soluble membranes; SI, sarcosyl-insoluble membranes.

of *tcpI*. The TCP-expressing phenotype of *V. cholerae* KP5.51 is consistent with the finding that *tcpI* encodes a protein that negatively regulates the synthesis of the major TCP subunit (51). Additional sequencing of DNA flanking *tagB*, not presented in this report, confirmed that *tagB* resided in the *tcp* gene cluster at the previously reported map position of *tcpI* (49). In light of these data, we propose that *tagB* be renamed *tcpI*.

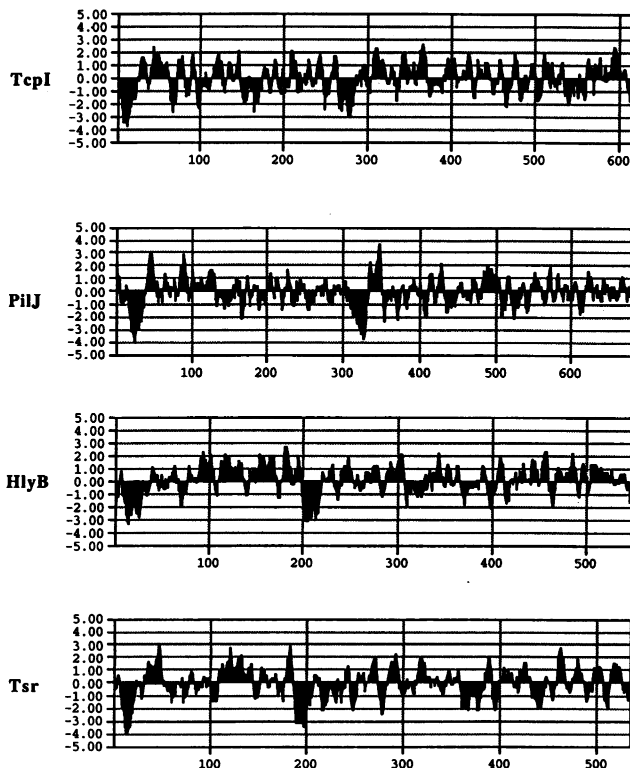

FIG. 5. The hydrophilicity profiles of TcpI, PilJ, HlyB, and Tsr. Areas with positive values represent hydrophilic amino acids, and areas with negative values represent hydrophobic amino acid residues. The numbers at the bottom of each panel refer to amino acid positions within the four proteins.

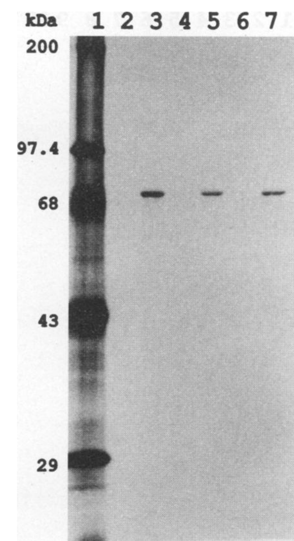

FIG. 6. SDS-PAGE fluorography of intrinsically radiolabeled proteins present in *E. coli* ER1451 cells following expression of *V. cholerae tcpI*. Lanes: 1, electrophoretic migration of radiolabeled molecular-weight-standard proteins; 2, fluorographic profile of *E. coli* cells bearing pBluescriptII KS$^+$; 3, gel pattern of *E. coli* cells bearing pTCPI-1; 4 and 6, profiles of *E. coli* cells bearing pBluescriptII KS$^+$ that were treated with 9% ethanol and 4 mmol of sodium azide, respectively; 5 and 7, protein profiles of *E. coli* cells bearing pTCPI-1 that were treated with 9% ethanol and 4 mmol of sodium azide, respectively. The numbers on the left indicate the molecular sizes of the radiolabeled protein standards.

The DNA sequence from the *tcpI*::Tn*phoA* fusion and the cloned *tcpI* gene revealed an 1,860-nucleotide open reading frame. Computer-aided searches of the National Center for Biotechnology Information data base revealed a striking similarity between TcpI and *E. coli* and *Salmonella* MCPs (5, 7, 21, 41). MCPs are integral inner membrane proteins that function as chemosensors, providing the bacterial cell with a means to respond to various environmental signals. MCP recognition of certain chemical signals results in the activation of a phosphorelay pathway that changes the bias of the flagellar motor, permitting the cell to swim toward attractants and away from repellents (6, 22). The similarity between TcpI and the MCPs is most notable in the HCD that functions in conjunction with CheW and CheA to modulate flagellar switching (20, 22, 29, 40). MCPs possess several other well-characterized domains: an amino-terminal hydrophobic leader sequence that is not cleaved by signal peptidase I, a globular periplasmic domain that binds specific ligands, a second hydrophobic transmembrane domain, and a linker region that transmits a ligand-binding-dependent signal to the highly conserved domain (1) present in the cytoplasmic portion of the protein. The cytoplasmic domain also contains two methylation domains (K1 and R1) that flank the signaling domain (1, 21, 41) which are responsible for controlling the adaptation response of the cell to chemical stimuli. The data presented in this report support the idea that TcpI possesses many of the structural characteristics of MCPs. Localization of TcpI to the inner membrane fraction of *E. coli* and *V. cholerae* and the lack of TcpI leader peptide cleavage indicate that TcpI is an integral inner membrane protein. Computer analysis predicts two membrane-spanning domains for TcpI that are spatially conserved with those found in the MCPs. These predictions are supported by preliminary studies employing Tn*phoA* and Tn*lacZ* as topology

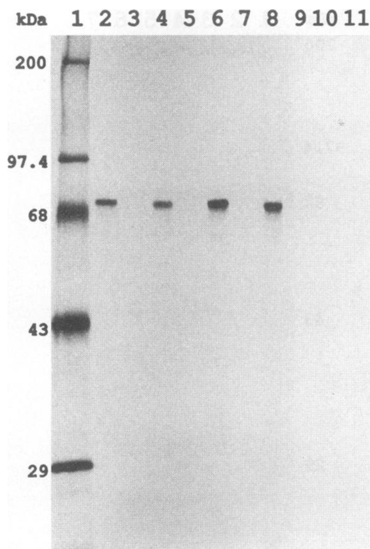

FIG. 7. SDS-PAGE fluorography of subcellular fractions isolated from *E. coli* ER1451 cells following T7-directed expression of *V. cholerae tcpI*. Lanes: 1, electrophoretic migration of radiolabeled molecular-weight-standard proteins; 2 and 3, whole-cell protein patterns of *E. coli* bearing pTCPI-1 (*tcpI* expressing cells) and pBluescript-II KS$^+$ (control cells), respectively; 4 and 5, fluorographic profiles of soluble fraction proteins isolated from *tcpI*-expressing cells and control cells, respectively; 6 and 7, gel patterns of total membrane proteins isolated from *tcpI*-expressing and control cells, respectively; 8 and 9, protein patterns of sarcosyl-soluble membrane preparations isolated from *tcpI*-expressing cells and control cells, respectively; 10 and 11, demonstration of absence of TcpI in sarcosyl-insoluble membrane preparations isolated from *tcpI*-expressing cells and control cells, respectively.

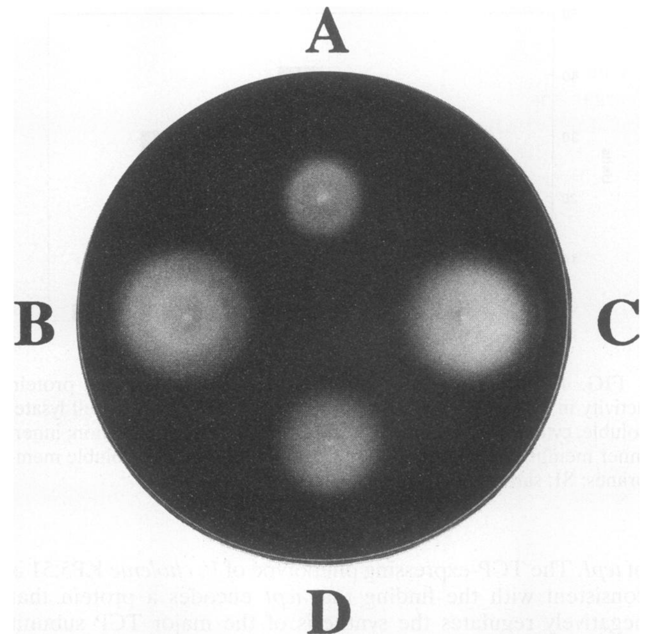

FIG. 8. Swarm plate analysis of *V. cholerae tcpI*::Tn*phoA* mutants. The *V. cholerae* strains are O395 (A), KP5.51 (*tcpI*::Tn*phoA*) (B), KP7.74 (*tcpI*::Tn*phoA*) (C), and KP1.17 (*toxR12*) (D).

probes. Limited analysis of *tcpI*::Tn*lacZ* and *tcpI*::Tn*phoA* insertions indicate that TcpI possesses a cytoplasmic domain and a periplasmic domain. The cytoplasmic domains of MCPs contain Gln-Gln and Gln-Glu pairs that serve as methyl acceptors in a process that modulates the memory component of chemotaxis. TcpI also possesses Gln-Gln and Gln-Glu pairs in the putative cytoplasmic domain. Future studies will attempt to discern if these residues are capable of being methylated in vibrio cells.

The periplasmic amino-terminal domains of MCPs bind attractants in the form of various sugars and amino acids and recognize repellents (weak acids, various alcohols, hydrophobic amino acids, certain aromatics, indole, nickel, and cobalt). The ability of MCPs to recognize these attractants and repellents leads to an alteration of the smooth swimming and tumbling frequency that is characteristic of bacterial chemotaxis (20, 22). The putative periplasmic domain of TcpI showed little homology to the periplasmic ligand-binding domains of the MCPs present in the National Center for Biotechnology Information data base, making it impossible at this point to discern by analogy which signal(s) TcpI may recognize. Our finding that TcpI resembles a class of procaryotic chemosensors may help to elucidate the signal transduction pathway responsible for ToxR-ToxT-directed activation of pilus biogenesis. Key to this understanding will be the identification of the chemical signal(s) that TcpI recognizes. The environmental signals that are known to modulate TCP biogenesis, i.e., pH, osmolarity, oxygen tension, and amino acids such as asparagine and aspartic acid, would seem likely candidates for such signals.

Growth of *V. cholerae* under conditions that promote TAG expression leads to diminished vibrio swarming. Similar results are seen when OrfI, an MCP homolog of unknown function from the transposon Tn*1721*, is synthesized at high levels in *E. coli* cells. That is, overexpression of *orfI* results in diminished *E. coli* swarming in semisolid agar (2). Insertional inactivation of *tcpI* (*tcpI*::Tn*phoA*; strains KP5.55 and KP7.74) results in a reduction in the ToxR regulon-mediated dampening of *V. cholerae* swarm plate activity. Although the mutations in KP5.51 and KP7.74 are transposon induced, we believe that the increased swarming of these strains is specific to *tcpI*. Our basis for this statement is twofold: *tcpI* is transcribed as a monocistronic message in the opposite direction of the *tcp* genes and open reading frames depicted in Fig. 1, and deletion mutations in *acfB*, a gene encoding an MCP homolog in the *V. cholerae acf* gene cluster, yield cells with the same swarm plate phenotype as vibrios carrying *tcpI* mutations (data not shown). Complementation of the mutations in KP5.51 and KP7.74 by using multicopy plasmids bearing *tcpI* will determine the specificity of the *tcpI* mutations more directly. The results of the swarm plate assays provide evidence for a mechanism whereby *V. cholerae* can control motility at the site of infection. Presumably, during periods of maximal TCP production and microcolony formation, motility would be an undesirable property. The ability of TcpI to decrease vibrio motility may be explained by the presence of the HCD. Increased synthesis of TcpI, which contains an HCD, may somehow disrupt the *V. cholerae* chemotaxis signal transduction pathway. The ability of the HCD within TcpI to interact with vibrio CheW and CheA homologs would provide a mechanism whereby these regulatory proteins are sequestered away from the chemotaxis machinery. We are currently designing experiments aimed at directly demonstrating TcpI interactions with *V. cholerae* and *E. coli* chemotaxis regulatory proteins to test this hypothesis.

Amino acid similarity searches indicate that TcpI is also related to HlyB, a *V. cholerae* protein involved in hemolysin

secretion. Although the greatest degree of similarity between these two vibrio proteins lies in the putative HCD regions, the role of HlyB in protein secretion may provide additional clues regarding the function of TcpI in TCP biogenesis. Computer analysis of the HlyB primary amino acid sequence predicts a membrane topology for HlyB that is similar to that of the MCPs and TcpI. Mutations in HlyB prevent the translocation of HlyA from the cytoplasm of the cell through the outer membrane (3). If HlyB and TcpI are functional homologs, then it follows that TcpI may play a role in regulating the transport of TcpA from the periplasmic space to the vibrio outer membrane.

In this report, we also show that TcpI is related to PilJ, a *P. aeruginosa* MCP homolog involved in a novel form of flagellum-independent surface translocation termed twitching motility (8, 9, 23). Twitching motility is linked with the presence of thin pili on a number of bacterial species (23). The retractile pili of *P. aeruginosa* provide the organism with a means of spreading across a solid surface (8). PilJ, in conjunction with three Che protein homologs (PilG and PilH [CheY homologs] and PilI [a CheW homolog]), represents a signal transduction pathway that controls *P. aeruginosa* pilus biosynthesis and twitching motility (9). While the TcpI-PilJ homology reinforces the similarities between the biogenesis pathways of these two type 4 pili, there are some notable differences between the two systems. Mutations in *pilJ* abolish both pilus formation and twitching motility. Mutations in *tcpI* result in the expression of a fully functional pilus. The *Pseudomonas pilG*, *pilH*, *pilI*, and *pilJ* gene cluster is unlinked to the gene encoding the pilus structural subunit (*pilA*). The *tcpI* gene lies close to the pilus structural subunit gene (*tcpA*), and to date, no *V. cholerae* Che protein homologs have been identified within the *tcp* gene cluster.

Previous studies suggest that TcpI affects TCP biogenesis by negatively regulating the synthesis of TcpA (51). This effect may be indirect since computer algorithms fail to detect any conventional DNA-binding domains within TcpI. One possibility would be for TcpI to modulate the activity of a protein that controls *tcpA* expression. We believe TcpH (Fig. 1) is a likely candidate for such a protein since it has previously been assigned a role in the positive regulation of TcpA synthesis (51). A detailed genetic analysis of *tcpA* expression will be necessary to elucidate the role of TcpI and TcpH in the control of TcpA synthesis. The striking similarity between TcpI and bacterial MCPs implies that sensing of the local environment by TcpI may play a role in regulating TcpA synthesis. We find it intriguing that TcpI, whose expression is controlled by a regulatory cascade that responds to environmental signals (12), may also function as an environmental regulator of a *V. cholerae* virulence determinant. Experiments aimed at determining the ability of TcpI to be methylated, the nature of the environmental signal(s) TcpI responds to, the specific interaction of TcpI with putative *V. cholerae* CheA and CheW homolog proteins, and the role of TcpI in modulating motility and chemotaxis will provide further insight into the mechanisms utilized by *V. cholerae* to fine-tune the expression of virulence determinants.

The information presented in this report affords an opportunity to build on previous models regarding the role of the ToxR regulatory cascade in the physiology of intestinal colonization (10, 12, 42). At some unknown point in the infection process, possibly as the vibrios penetrate the mucus lining of the intestine, TAG are expressed in response to some unknown host signal(s). TAG expression would lead to the synthesis of proteins involved in pilus biogenesis but not necessarily in the surface expression of TCP, since TcpA synthesis is negatively regulated by TcpI. Once *V. cholerae* cells reach the correct anatomical site (epithelial surface?), signal transduction by TcpI in response to the appropriate environmental signal may eliminate the repression of TcpA, thereby promoting TCP assembly. The additional layer of regulation provided by TcpI may afford the vibrios a mechanism to fine-tune the expression of this critical colonization determinant such that efficient TCP biogenesis is limited to locations within the host that are suitable for stable microcolony formation. We further hypothesize that synthesis of TcpI represses vibrio swarming in vivo, further promoting localization of the bacterium at the most suitable intestinal microenvironment. It might be expected, therefore, that mutations in a gene (*tcpI*) affecting both TCP synthesis and swarming would render the vibrios less virulent. Interestingly, *V. cholerae tcpI* mutants are fully capable of colonizing the intestines of infant mice (45). This finding may have important implications with respect to utilizing the infant mouse model for detecting and/or characterizing subtle differences in the expression of *V. cholerae* colonization determinants. The lack of a colonization defect associated with *V. cholerae tcpI* mutants in the infant mouse model makes it impossible at this point in time to determine the precise role of TcpI in the overall physiology of intestinal colonization. It may be necessary to determine the colonization phenotype of *V. cholerae tcpI* mutants in alternative in vivo models (17) and/or in the natural host (via human challenge studies) to gain true insight regarding the role of TcpI in intestinal colonization.

ACKNOWLEDGMENTS

This work was supported by Public Health Services grant AI28502 from the National Institutes of Health and a grant from the Center for Excellence in Cancer Research, Treatment and Education, Louisiana State University Medical Center.

We thank A. Darzins, R. K. Taylor, and C. Gardel for sharing information prior to publication. We also thank V. DiRita and R. K. Taylor for helpful discussions.

REFERENCES

1. **Alley, M. R. K., J. R. Maddock, and L. Shapiro.** 1992. Polar localization of a bacterial chemoreceptor. Genes Dev. **6:**825–836.
2. **Allmeier, H., B. Cresnar, M. Greck, and R. Schmitt.** 1992. Complete nucleotide sequence of Tn*1721*: gene organization and a novel gene product with features of a chemotaxis protein. Gene **111:**11–20.
3. **Alm, R. A., and P. A. Manning.** 1990. Characterization of the *hlyB* gene and its role in the production of the El Tor haemolysin of *Vibrio cholerae* O1. Mol. Microbiol. **4:**413–425.
4. **Baudry, B., A. Fasano, J. M. Ketley, and J. B. Kaper.** 1992. Cloning of a gene (*zot*) encoding a new toxin produced by *Vibrio cholerae*. Infect. Immun. **60:**428–434.
5. **Bollinger, L., C. Park, S. Harayama, and G. L. Hazelbauer.** 1984. Structure of the Trg protein: homologies with and differences from other sensory transducers of *Escherichia coli*. Proc. Natl. Acad. Sci. USA **81:**3287–3291.
6. **Borkovich, K. A., N. Kaplan, J. F. Hess, and M. I. Simon.** 1989. Transmembrane signal transduction in bacterial chemotaxis involves ligand-dependent activation of phosphate group transfer. Proc. Natl. Acad. Sci. USA **86:**1208–1212.
7. **Boyd, A., K. Kendall, and M. I. Simon.** 1983. Structure of the serine chemoreceptor in *Escherichia coli*. Nature (London) **301:** 623–626.
8. **Bradley, D. E.** 1980. A function of *Pseudomonas aeruginosa* polar pili: twitching motility. Can. J. Microbiol. **26:**146–154.
9. **Darzins, A.** 1994. Characterization of a *Pseudomonas aeruginosa* gene cluster involved in pilus biosynthesis and twitching motility: sequence similarity to the chemotaxis proteins of enterics and the gliding bacterium *Myxococcus xanthus*. Mol. Microbiol. **11:**137–153.
10. **DiRita, V. J.** 1992. Coordinate control of virulence gene expression

by ToxR in *Vibrio cholerae*. Mol. Microbiol. **6**:451–458.
11. **DiRita, V. J., and J. J. Mekalanos.** 1991. Periplasmic interaction between two membrane regulatory proteins, ToxR and ToxS, results in signal transduction and transcriptional activation. Cell **64**:29–37.
12. **DiRita, V. J., C. Parsot, G. Jander, and J. J. Mekalanos.** 1991. Regulatory cascade controls virulence in *Vibrio cholerae*. Proc. Natl. Acad. Sci. USA **88**:5403–5407.
13. **DiRita, V. J., K. M. Peterson, and J. J. Mekalanos.** 1988. Regulation of cholera toxin synthesis, p. 355–376. *In* B. H. Inglewski and V. L. Clark (ed.), Molecular basis of pathogenesis. Academic Press, Inc., New York.
14. **Fasano, A., B. Baudry, D. W. Pumplin, S. S. Wasserman, B. D. Tall, J. M. Ketley, and J. B. Kaper.** 1991. *Vibrio cholerae* produces a second enterotoxin, which affects intestinal tight junctions. Proc. Natl. Acad. Sci. USA **88**:5242–5246.
15. **Filip, C., G. Fletcher, J. L. Wulff, and C. F. Earhart.** 1973. Solubilization of the cytoplasmic membrane of *Escherichia coli* by the ionic detergent sodium-lauryl sarcosinate. J. Bacteriol. **115**: 717–722.
16. **Freter, R., and P. C. M. O'Brien.** 1981. Role of chemotaxis in the association of motile bacteria with intestinal mucosa: fitness and virulence of nonchemotactic *Vibrio cholerae* mutants in infant mice. Infect. Immun. **34**:222–233.
17. **Freter, R., P. C. M. O'Brien, and M. M. S. Macsai.** 1981. Role of chemotaxis in the association of motile bacteria with intestinal mucosa: in vivo studies. Infect. Immun. **34**:234–240.
18. **Gardel, C. L., and J. J. Mekalanos.** 1994. Modus operandi of *Vibrio cholerae*: swim to arrive; stop to kill. The relationship among chemotaxis, motility and virulence. J. Cell. Biochem. **18A**:65.
19. **Guenzel, M. N., and L. J. Berry.** 1975. Motility as a virulence factor of *Vibrio cholerae*. Infect. Immun. **15**:539–548.
20. **Hazelbauer, G. L.** 1983. Sensory transduction in bacterial chemotaxis. Int. Rev. Cytol. **81**:33–70.
21. **Hazelbauer, G. L.** 1992. Bacterial chemoreceptors. Curr. Opin. Struct. Biol. **2**:505–510.
22. **Hazelbauer, G. L.** 1993. Bacterial motility and signal transduction. Cell **73**:15–22.
23. **Henrichsen, J.** 1983. Twitching motility. Annu. Rev. Microbiol. **37**:81–93.
24. **Herrington, D. A., R. H. Hall, G. A. Losonsky, J. J. Mekalanos, R. K. Taylor, and M. M. Levine.** 1988. Toxin, toxin-coregulated pilus and the ToxR regulon are essential for *Vibrio cholerae* pathogenesis in human volunteers. J. Exp. Med. **168**:1487–1492.
25. **Higgins, D. E., E. Nazareno, and V. J. DiRita.** 1992. The virulence gene activator ToxT from *Vibrio cholerae* is a member of the AraC family of transcriptional activators. J. Bacteriol. **174**:6974–6980.
26. **Kaufman, M. R., C. E. Shaw, I. D. Jones, and R. K. Taylor.** 1993. Biogenesis and regulation of the *Vibrio cholerae* toxin-coregulated pilus: analogies to other virulence factor secretory systems. Gene **126**:42–49.
27. **Kaufman, M. R., J. M. Seyer, and R. K. Taylor.** 1991. Processing of TCP pilin by TcpJ typifies a common step intrinsic to a newly recognized pathway of extracellular protein secretion by gram-negative bacteria. Genes Dev. **5**:1834–1846.
28. **Kelley, J. T., and C. D. Parker.** 1981. Identification and preliminary characterization of *Vibrio cholerae* outer membrane proteins. J. Bacteriol. **145**:1018–1024.
29. **Liu, J., and J. S. Parkinson.** 1991. Genetic evidence for interaction between the CheW and Tsr proteins during chemoreceptor signaling by *Escherichia coli*. J. Bacteriol. **173**:4941–4951.
30. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
31. **McBride, M. J., T. Kohler, and D. R. Zusman.** 1992. Methylation of FrzCD, a methyl-accepting taxis protein of *Myxococcus xanthus*, is correlated with factors affecting cell behavior. J. Bacteriol. **174**:4246–4257.
32. **Mekalanos, J. J.** 1985. Cholera toxin: genetic analysis, regulation, and role in pathogenesis. Curr. Top. Microbiol. Immunol. **118**:97–118.
33. **Miller, J. F., J. J. Mekalanos, and S. Falkow.** 1989. Coordinate regulation and sensory transduction in the control of bacterial virulence. Science **243**:916–922.
34. **Miller, V. L., and J. J. Mekalanos.** 1984. Synthesis of cholera toxin is positively regulated at the transcriptional level by *toxR*. Proc. Natl. Acad. Sci. USA **81**:3471–3475.
35. **Miller, V. L., and J. J. Mekalanos.** 1988. A novel suicide vector and its use in construction of insertion mutations: osmoregulation in *Vibrio cholerae* of outer membrane proteins and virulence determinants require *toxR*. J. Bacteriol. **170**:2575–2583.
36. **Miller, V. L., R. K. Taylor, and J. J. Mekalanos.** 1987. Cholera toxin transcriptional activatory ToxR is a transmembrane DNA binding protein. Cell **48**:271–279.
37. **Ogierman, M. A., S. Zabihi, L. Mourtzios, and P. A. Manning.** 1993. Genetic organization and sequence of the promoter-distal region of the *tcp* gene cluster of *Vibrio cholerae*. Gene **126**:51–60.
38. **Osborne, M. J., J. E. Gander, E. Parisi, and J. Carson.** 1972. Mechanism of assembly of the outer membrane of *Salmonella typhimurium*. J. Biol. Chem. **247**:3962–3972.
39. **Palva, E. T., T. R. Hirst, S. J. S. Hardy, J. Holmgren, and L. Randall.** 1981. Synthesis of a precursor to the B subunit of heat-labile enterotoxin in *Escherichia coli*. J. Bacteriol. **146**:325–330.
40. **Parkinson, J. S.** 1993. Signal transduction schemes of bacteria. Cell **73**:857–871.
41. **Parkinson, J. S., and E. C. Kofoid.** 1992. Communication modules in bacterial signaling proteins. Annu. Rev. Genet. **26**:71–112.
42. **Parsot, C., and J. J. Mekalanos.** 1990. Expression of ToxR, the transcriptional activator of virulence factors in *Vibrio cholerae*, is modulated by the heat shock response. Proc. Natl. Acad. Sci. USA **87**:9898–9902.
43. **Parsot, C., and J. J. Mekalanos.** 1991. Expression of *Vibrio cholerae* aldehyde dehydrogenase is under the control of ToxR, the cholera toxin transcriptional activator. J. Bacteriol. **173**:2842–2851.
44. **Peek, J. A., and R. K. Taylor.** 1992. Characterization of a periplasmic thiol:disulfide interchange protein required for the funtional maturation of secreted virulence factors of *Vibrio cholerae*. Proc. Natl. Acad. Sci. USA **89**:6210–6214.
45. **Peterson, K. M., and J. J. Mekalanos.** 1988. Characterization of the *Vibrio cholerae* ToxR regulon: identification of novel genes involved in intestinal colonization. Infect. Immun. **56**:2822–2829.
46. **Rice, M. S., and F. W. Dahlquist.** 1991. Sites of deamidation and methylation in Tsr, a bacterial chemotaxis sensory transducer. J. Biol. Chem. **266**:9746–9753.
47. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74**:5463–5467.
48. **Schnaitman, C. A.** 1970. Examination of the protein composition of the cell envelope of *Escherichia coli* by polyacrylamide gel electrophoresis. J. Bacteriol. **104**:882–889.
49. **Shaw, C., K. M. Peterson, J. J. Mekalanos, and R. K. Taylor.** 1988. Genetic studies of *Vibrio cholerae* TCP biogenesis, p. 5–17. *In* Y. Takeda and R. B. Sack (ed.), Advances in research on cholera and related diarrheas. KTK Science Publishers, Tokyo.
50. **Tabor, S., and C. C. Richardson.** 1985. A bacteriophage T7 RNA polymerase/promoter system for controlled expression of specific genes. Proc. Natl. Acad. Sci. USA **82**:1074–1078.
51. **Taylor, R. K.** 1989. Genetic studies of enterotoxin and other potential virulence factors of *Vibrio cholerae*, p. 309–329. *In* D. A. Hopwood and K. F. Chater (ed.), Genetics of bacterial diversity. Academic Press Ltd., London.
52. **Taylor, R. K., C. Manoil, and J. J. Mekalanos.** 1989. Broad-host range vectors for delivery of Tn*phoA*: use in genetic analysis of secreted virulence determinants of *Vibrio cholerae*. J. Bacteriol. **171**:1870–1878.
53. **Taylor, R. K., V. L. Miller, D. B. Furlong, and J. J. Mekalanos.** 1987. Use of *phoA* gene fusions to identify a pilus colonization factor coordinately regulated with cholera toxin. Proc. Natl. Acad. Sci. USA **84**:2833–2837.
54. **Trucksis, M., J. E. Galen, J. Michalski, A. Fasano, and J. B. Kaper.** 1993. Accessory cholera enterotoxin (Ace), the third member of a *Vibrio cholerae* virulence cassette. Proc. Natl. Acad. Sci. USA **90**:5267–5271.
55. **von Heigne, G.** 1985. A new method for predicting signal sequence cleavage sites. Nucleic Acids Res. **14**:4683–4690.