Proc. Natl. Acad. Sci. USA
Vol. 95, pp. 730–734, January 1998
Microbiology

TcpP protein is a positive regulator of virulence gene expression in *Vibrio cholerae*

(transcription activation/fimbriae/pathogenesis/toxin-coregulated pilus)

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ABSTRACT **The production of several virulence factors in *Vibrio cholerae* O1, including cholera toxin and the pilus colonization factor TCP (toxin-coregulated pilus), is strongly influenced by environmental conditions. To specifically identify membrane proteins involved in these signal transduction events, we examined a transposon library of *V. cholerae* generated by Tn*bla* mutagenesis for cells that produce TCP when grown under various nonpermissive conditions. To select for TCP-producing cells we used the recently described bacteriophage CTXΦ-Kan, which uses TCP as its receptor and carries a gene encoding resistance to kanamycin. Among the isolated mutants was a transposon insertion in a gene homologous to *nqrB* from *Vibrio alginolyticus*, which encodes a subunit of a Na^+-translocating NADH:ubiquinone oxidoreductase, and *tcpI*, encoding a chemoreceptor previously implicated in the negative regulation of TCP production. A third transposon mutant had an insertion in *tcpP*, which is in an operon with *tcpH*, a known positive regulator of TCP production. However, TcpP was shown to be essential for TCP production in *V. cholerae*, as a *tcpP*-deletion strain was deficient in pili production. The amino-terminal region of TcpP shows sequence homology to the DNA-binding domains of several regulatory proteins, including ToxR from *V. cholerae* and PsaE from *Yersinia pestis*. Like ToxR, TcpP activates transcription of the *toxT* gene, an essential activator of *tcp* operon transcription. Furthermore, TcpH, with its large periplasmic domain and inner membrane anchor, has a structure similar to that of ToxS and was shown to enhance the activity of TcpP. We propose that TcpP/TcpH constitute a pair of regulatory proteins functionally similar to ToxR/ToxS and PsaE/PsaF that are required for *toxT* transcription in *V. cholerae*.**

Vibrio cholerae is a Gram-negative bacterium that causes the diarrheal disease cholera. To establish infection and cause disease, *V. cholerae* must express a variety of virulence factors, including cholera toxin (CT) and colonization factors such as the toxin-coregulated pilus (TCP). Expression of CT and TCP is strongly influenced by environmental stimuli, including temperature, pH, osmolarity, and composition of the growth medium (1–3). The mechanisms by which environmental conditions affect the coordinate expression of virulence factors by *V. cholerae* remain poorly understood but certainly involve transcriptional regulation mediated by several regulatory proteins (4, 5). The current model for virulence regulation is that of a cascade in which ToxR controls expression of ToxT, which itself is a transcriptional activator and directly controls expression of several virulence genes (5). ToxR is an inner membrane protein that contains a cytoplasmic DNA-binding domain with homology to the OmpR subfamily of the regulator proteins of the two-component signal transduction family (6). The periplasmic domain of ToxR is thought to interact with another transmembrane regulatory protein, ToxS, that stimulates its activity (7). ToxR, in conjunction with ToxS, regulates transcription of the *toxT* gene as well as a set of outer membrane proteins. ToxT is an AraC-like transcriptional activator that activates transcription of several genes, including *ctx* and *tcpA*, the latter encoding the structural subunit of TCP (8).

We aimed to identify proteins that are involved in the regulation of virulence factors by environmental stimuli in *V. cholerae*. We used the transposon Tn*bla* (9) to concentrate on the subset of those proteins that are membrane associated or secreted. Mutant bacteria that express TCP under noninducing conditions were selected for by using a kanamycin (Kan) resistance-encoding derivative of the recently described bacteriophage CTXΦ, which uses TCP as its receptor (10). The results presented here indicate that TcpP, a protein encoded by a previously identified ORF in the *tcp* cluster, is an important regulator of virulence gene transcription in *V. cholerae*.

MATERIALS AND METHODS

Bacterial Strains and Growth Media. *Escherichia coli* DH5α (BRL) was used for cloning experiments, and *E. coli* SM10λpir (3) was used to transfer plasmids to *V. cholerae* by conjugation. *V. cholerae* strain O395-N1 (11) was used as the host for tansposon mutagenesis. *E. coli* and *V. cholerae* strains were maintained at −70°C in Luria–Bertani (LB) medium containing 20% (vol/vol) glycerol. Antibiotics were used at the following concentrations: ampicillin (Amp), 50 μg/ml; streptomycin (Sm), 100 μg/ml; Kan, 50 μg/ml; chloramphenicol (Cam), 50 μg/ml for *E. coli* and 5 μg/ml for *V. cholerae*. For TCP-producing conditions, *V. cholerae* strains were grown in LB broth (pH 6.5) at 30°C for 18 hr with aeration. For conditions not favoring pili production, cells were grown in LB at pH 8.5 at 30°C, in LB containing 20 mM procaine at 30°C, or in LB at 37°C.

Phage Transduction. Phage particles of a derivative of CTXΦ (10) that carries a gene encoding resistance to Kan were prepared from the culture supernatants of *V. cholerae* strain Peru-2 (pCtx-Kan) after overnight growth at 30°C by filtration through a 0.2-μm-pore filter unit. Recipient TCP-producing *V. cholerae* strains were grown under various conditions and incubated with an equal volume of phage at room temperature for 30 min and plated on LB plates containing the appropriate antibiotics.

Genetic Methods. Random insertions of Tn*bla* into the chromosome of *V. cholerae* were accomplished through the use of pWL10, a derivative of pGP704 (3) carrying a copy of Tn*bla*. Mutant strains were screened for single transposon insertions by Southern blotting (12). Fragments carrying the insertion

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This paper was submitted directly (Track II) to the *Proceedings* office.
Abbreviations: CT, cholera toxin; TCP, toxin-coregulated pilus; Kan, kanamycin.
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sequences as well as adjacent chromosomal junctions were amplified by inverse PCR using primers homologous to *bla* sequences. The PCR products were then isolated from agarose gels and used directly in sequencing reactions (DNA Core Facility, Harvard Medical School) using the same primers.

Isolation of *V. cholerae* Mutants Derepressed for Pili Production. CTXΦ phage particles (10) carrying a gene encoding resistance to Kan (CTXΦ-Kan) were used to select for *V. cholerae* mutants derepressed for the expression of TCP at normally nonpermissive conditions. Pools of *V. cholerae* colonies carrying inserts of Tn*bla* were grown in LB medium with a starting pH of 8.5 or in LB supplemented with 20 mM procaine. TCP-producing cells were isolated on Kan-containing plates after phage transduction. Kan-resistant colonies isolated after the initial selection were pooled, grown in the absence of Kan overnight, and streaked to obtain single colonies. Loss of the phage was screened for by replica plating colonies onto Kan-containing media. Individual Kan-sensitive colonies were then retested for TCP production under the noninducing conditions by phage transduction.

Generation of *V. cholerae* Strains Carrying Specific Chromosomal Mutations. The desired mutation were introduced into the chromosome of *V. cholerae* strains by using the suicide plasmid vectors pCVD442 (13) or pKAS32 or pKAS46 (14). Mutant alleles were first cloned in *E. coli* strain SM10λpir and then conjugated into *V. cholerae*. The appropriate counter-selections were used to isolate *V. cholerae* strains that had lost the vector DNA followed by screening for the mutation by PCR. To generated the Δ*tcpP* strain, a PCR product carrying the entire *tcpP* gene as well as flanking sequences was inserted into pCR2.1 (Invitrogen). After passage through a dam^- *E. coli* strain, a 426-bp *Bcl*I restriction fragment internal to *tcpP* was deleted, resulting in an in-frame deletion in *tcpP*. The resulting DNA fragment was then inserted into pCVD442 and introduced into the *V. cholerae* chromosome by homologous recombination. The Tn*bla* mutation was reintroduced into a wild-type *V. cholerae* strain by cloning the PCR product from the initial transposon mutant into pKAS46 followed by conjugation into *V. cholerae*.

Construction of *toxT*::*lacZ* Reporter Strains. The *toxT* gene and flanking sequences were cloned from pGJ40 (15) as a *Sna*BI–*Spe*I fragment into pKAS32 digested with *Eco*RV and *Xba*I (pKAS32-T). The *lacZ* gene was amplified from pCH110 (Pharmacia) by using primers with added *Hin*dIII sites and inserted into the *Hin*dIII site of *toxT* after a partial digestion of pKAS32-T leading to a ToxT-LacZ fusion protein under the control of the *toxT* promoter. This construct was introduced into the chromosome of several *V. cholerae* strains.

β-Galactosidase Assays. *V. cholerae* cells carrying various plasmid constructs were grown overnight in LB medium at 30°C in the presence or absence of arabinose and assayed for β-galactosidase by using a modified version of the method described by Miller (16).

RESULTS

Isolation and Characterization of Mutant *V. cholerae* Strains. To identify membrane or secreted proteins that, when mutated, allow expression of TCP in *V. cholerae* under noninducing conditions, random mutagenesis was performed with Tn*bla*. Tn*bla* carries a promoterless and signal sequence-less β-lactamase gene that will result in ampicillin-resistant cells only if it is fused to a gene for an expressed membrane or secreted protein (9). A Kan-marked derivative of the CTXΦ phage (10) was used to select for TCP-producing cells after growth under noninducing conditions, such as elevated pH or addition of procaine. We had previously observed that the addition of local anesthetics such as procaine to the growth medium strongly inhibited transcription of genes controlled by the ToxR/ToxT regulatory cascade (J.J.M. and R. Taylor, unpublished results), including *tcpA*.

Three mutant strains that had single Tn*bla* insertions and were derepressed for TCP production were isolated. The chromosomal genes interrupted by the Tn*bla* insertions were determined by cloning insertion junctions and DNA sequencing. One mutant strain had a transposon insertion in a gene highly homologous to *nqrB* from *Vibrio alginolyticus* (17, 18). This gene is believed to encode a subunit of an enzyme involved in production of an electrochemical gradient of sodium ions during aerobic respiration. The other mutant strains had insertions in two genes, *tcpI* and *tcpP*, that were previously implicated in the regulation of TCP and lie within the *tcp* gene cluster (Fig. 1*A*). A *tcpI* mutation has previously been described, and its gene product was implicated in the negative regulation of TCP production in *V. cholerae* (19). No mutations in *tcpP* had previously been reported, although the gene immediately downstream of *tcpP* is *tcpH*, whose gene product was suggested to be a positive regulator of TCP production (19). Furthermore, recent data suggested that spontaneous CT^- TCP^- mutants of *V. cholerae* have frameshift mutations in *tcpH*, also supporting a role for TcpH in the regulation of the ToxR regulon (27).

Characterization of *V. cholerae* Strains Carrying Mutations in *tcpP*. To verify that the deregulated TCP-producing phenotype is linked to the transposon insertion, the *tcpP*::Tn*bla* mutation was reintroduced into a wild-type strain, where it showed a pattern of elevated TCP production in the presence of procaine similar to that of the original mutant (data not shown). Because we could not distinguish whether the TcpP-Bla fusion protein was active, inactive, or constitutive for TcpP activity, we decided to construct a null mutation in *tcpP*. A *V. cholerae* strain carrying an in-frame deletion mutant in *tcpP* was constructed (Δ*tcpP*) and was found to be resistant to the

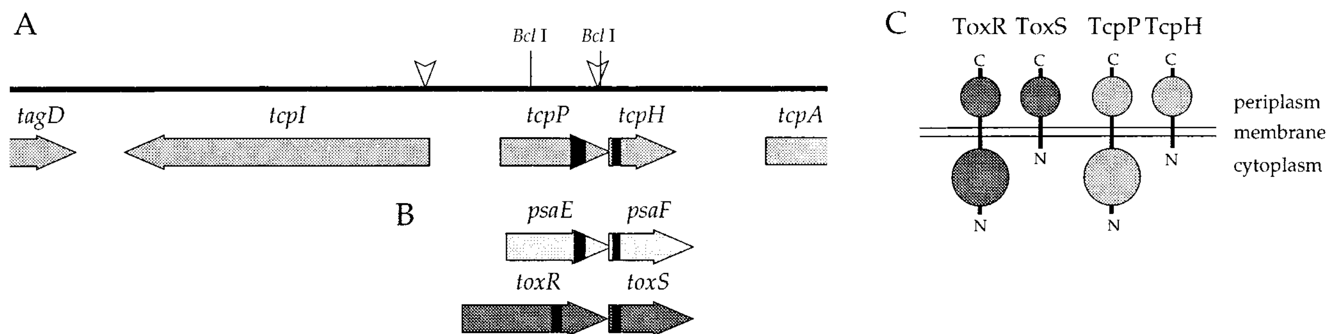

FIG. 1. (*A*) Genomic map of the region in the *tcp* gene cluster affected by two independent transposon insertions (arrowheads). The *Bcl*I sites show the extent of the deletion in the Δ*tcpP* strain. (*B*) Comparison of the *tcpP* and *tcpH* genes to *psaE* and *psaF* from *Yersinia pestis* (20) and *toxR* and *toxS* from *V. cholerae* (6, 7). Arrows indicate the direction and sizes of the ORFs. Sequences encoding hydrophobic regions representing potential transmembrane domains are shown in black. (*C*) Proposed membrane topology of ToxR, ToxS, TcpP, and TcpH. Carboxyl (C) and amino (N) termini of the proteins are indicated.

Table 1. Gene complementation

Strain	Growth in arabinose 0%	Growth in arabinose 0.04%
Δ*tcpP*	—	
Δ*tcpP* (pBAD-P)	—	++
Δ*tcpP* (PBAD-P:bla)	—	++
Δ*tcpP* (pBAD-T)	—	++
Δ*tcpP* (pBAD-R)	—	—
Δ*toxR*	—	
Δ*toxR* (pBAD-R)	—	++
Δ*toxR* (pBAD-T)	—	++
Δ*toxR* (pBAD-P)	—	+
Δ*toxT*	—	
Δ*toxT* (pBAD-T)	—	++
Δ*toxT* (pBAD-R)	—	—
Δ*toxT* (pBAD-P)	—	—

V. cholerae strains with chromosomal deletions in *tcpP*, *toxR*, or *toxT* carrying plasmids with these genes under an arabinose-inducible promoter were grown overnight in LB medium at 30°C without or with 0.04% arabinose, followed by transduction. A + indicates at least 50 Kan-resistant colonies, whereas a ++ indicates a confluent lawn of Kan-resistant colonies in 25 μl of plated culture.

CTXΦ-Kan phage, indicating that no functional pili are produced (Table 1). However, this strain was fully complemented by introducing a plasmid containing the *tcpP* gene under an arabinose-inducible promoter (pBAD-P) only in the presence of arabinose (Table 1). As the *tcpP*::Tn*bla* insertion occurred in the very carboxyl-terminal region of TcpP (Fig. 1), it is possible that the TcpP-Bla fusion molecule remains functional. In fact, the cloned *tcpP*::Tn*bla* gene can complement the Δ*tcpP* mutation (Table 1). Additionally, the Δ*tcpP* mutant strain was complemented for TCP production by a *toxT*-expressing plasmid (pBAD-T) (Table 1).

TcpP Is a Positive Regulator. Sequence alignment of TcpP with proteins in the databases revealed similarities between the amino-terminal region of TcpP and the DNA-binding domains of several regulatory proteins (Fig. 2), including PsaE from *Y. pestis* (20), HilA from *S. typhimurium* (21), and ToxR from *V. cholerae* (6). Furthermore, like ToxR, TcpP has a hydrophobic segment (from Ile-141 to Gln-169) that likely defines a transmembrane helix, suggesting it may have a topology similar to that of ToxR. These similarities suggested that TcpP was likely to be a transcriptional regulator. To investigate at which level in the regulatory cascade of TCP production in *V. cholerae* TcpP exerts its effect, plasmids expressing *toxT*, *toxR*, or *tcpP* were introduced into various *V. cholerae* deletion strains and assayed for TCP production by phage transduction (Table 1). As expected, all genes complemented their respective chromosomal mutations. In addition, *toxT*, when expressed from an inducible promoter, led to pili production regardless of which regulatory gene was deleted in the chromosome, consistent with previous results indicating that ToxT is hierarchically the last regulator in the ToxR–ToxT cascade (22). Furthermore, the *tcpP*-expressing plasmid weakly complemented a *toxR*-deletion strain but not a *toxT*-deletion strain for TCP production (Table 1). In contrast, the *toxR*-expressing plasmid did not complement a *tcpP*-deletion or a *toxT*-deletion strain (Table 1).

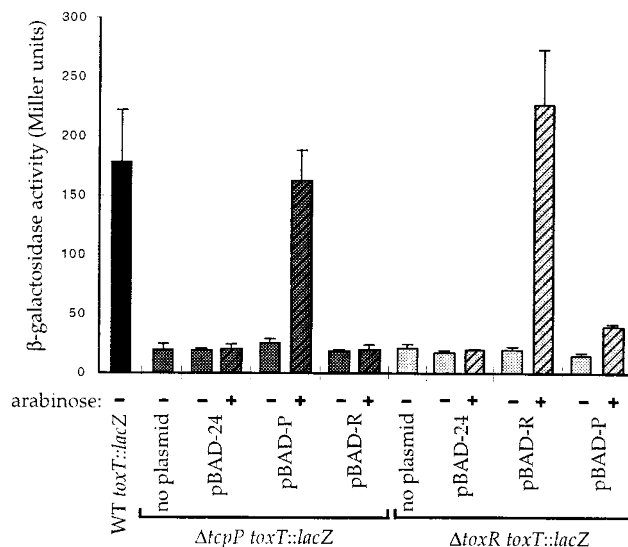

FIG. 3. β-Galactosidase activities of *V. cholerae* strains carrying a *toxT*::*lacZ* reporter construct in the chromosome with plasmids carrying the *tcpP* or *toxR* gene under arabinose-inducible promoters. Arabinose was added to a final concentration of 0.04%.

TcpP Is Involved in Transcription of *toxT*. To investigate whether TcpP is involved in the transcription of *toxT*, a *toxT*::*lacZ* fusion reporter construct was generated and introduced into the chromosome of various *V. cholerae* strains, and then plasmids carrying different complementing genes were introduced. The β-galactosidase expression levels of these strains are summarized in Fig. 3. As expected, the Δ*toxR* strain expressed very low β-galactosidase activity, but was complemented by the *toxR*-carrying plasmid. Similarly, the Δ*tcpP* strain expressed almost no β-galactosidase activity but was fully complemented by a *tcpP*-carrying plasmid. As in a Δ*toxR* strain, when the plasmid pRSI-2, carrying the *toxT* promoter (−172 to +45 relative to the ToxR-dependent start site) in front of a promoterless *lacZ* gene (23), was introduced into the Δ*tcpP* strain, β-galactosidase expression compared with the parental strain was reduced (data not shown). This finding suggests that TcpP acts directly upstream of the *toxT* gene.

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TcpP    1            MGYVRVIYQFPDNLWWNECSNQVYYAQDPMKPE..RLIGTPSIMQAKLLKILCE
PsaE    1                             MSHCVVLNKLESVLIIGD.SRY..ALSKNEVLLLECLYL
HilA    1            MPHFNPVPVSNKKFVFDDFI..LNMDGSLVRSE..KKVNIPPK.EYAVLVILLE
ToxR    1   MFGLGHNSKEISMSHIGTKFILAEKFTFDPLSNTLIDKEDSEEIIRLGSNESRILWLLAQ

TcpP   53   YHPSPCPNDQIIKALWPH.GF.ISSESLTQAIKRTRDFLN.D.EHKTLIENVKLQGYRIN
PsaE   37   RAGDVISHDELLTTCWPDR..VVSPTSLPVAIKHIRDVFRKITRS.EVIKTYKNEGYSYQ
HilA   50   AAGKIVSKNTLLDQVWGDA..EVNEESLTRCIYALRRILSEDKEHR.YIETLYGQGYRFN
ToxR   61   RPNEVISRNDLHDFVWREQGFEVDDSSLTQAISTLRKMLKDSTKSPQYVKTVPKRGYQL.

TcpP  109   I.IQVIVSENIVDEAD.CS..QKKSVKERIKIEWGKINVVPYLVFSALYVALLPVI
PsaE   94   KDSVLIIIDDGSTEKESHSAAYTRKEKPDIPIKLVGLQILSHLNSTFFIAIMMVII
HilA  107   R.PVVVVSPPAPQPTTHTLAILPFQMQDQVQSESLHYSIVKGLSQYAPF.GLSVLP
ToxR  120   IARVETVEEEMARENEAAHDIS....QPESVNEYAESSSVPSSATVVNTPQPANVV
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FIG. 2. Amino acid sequence alignment of the amino-terminal region of the *V. cholerae* TcpP protein and the proteins PsaE from *Y. pestis* (20), HilA from *Salmonella typhimurium* (21), and ToxR from *V. cholerae* (6). Identical amino acids are shown in black boxes, whereas gray boxes indicate similar amino acid residues.

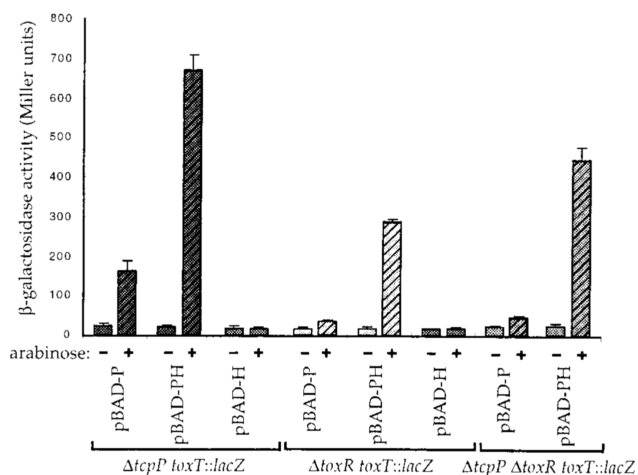

FIG. 4. β-Galactosidase activities of *V. cholerae* strains carrying a *toxT*::*lacZ* reporter construct in the chromosome with plasmids carrying the *tcpP* and/or *tcpH* genes under arabinose-inducible promoters. Arabinose was added to a final concentration of 0.04%.

Analogous to the phage transduction results, the plasmid encoding the *tcpP* gene partially complemented a *toxR* deletion strain for *toxT* transcription (Fig. 3). These data indicate that while both ToxR ad TcpP can partially activate the *toxT* promoter, both are required to get efficient activation.

TcpH Enhances the Activity of TcpP. Sequence comparison of TcpH, encoded by the gene downstream and possibly in an operon with *tcpP*, did not reveal any significant homology with other known proteins. However, TcpH shows an amino-terminal hydrophobic region that might represent a membrane anchor; consistent with its putative membrane protein structure, an active PhoA fusion to TcpH has been previously reported (19). A similar structure is found in ToxS from *V. cholerae* as well as PsaF from *Y. pestis*, and these proteins have been shown to enhance the activities of the transcriptional activators ToxR and PsaE, respectively. Both *toxS* and *psaF* are located immediately downstream and in an operon with their respective regulators (Fig. 1*B*). This similarity prompted us to investigate whether TcpH can enhance the activity of TcpP. Comparison of the two constructs pBAD-P and pBAD-PH showed that the presence of *tcpH* leads to higher β-galactosidase activities when the *toxT*::*lacZ* reporter construct is assayed in several *V. cholerae* deletion strains (Fig. 4). However, pBAD-H, which was constructed by deleting the *Bcl*I DNA fragment internal to *tcpP* from the pBAD-PH construct, did not activate the *toxT*::*lacZ* reporter construct (Fig. 4). Furthermore, less arabinose was required for complementation of a Δ*tcpP* mutant strain for TCP production by the plasmid expressing *tcpP* and *tcpH* compared with the plasmid expressing only *tcpP* (data not shown).

ToxR/S and TcpP/H Act Synergistically. The plasmid pVJ21 (24), expressing *toxR* and *toxS* constitutively, weakly increased the β-galactosidase activities in a Δ*tcpP* (data not shown) and a Δ*tcpP* Δ*toxR toxT*::*lacZ* reporter strain (Table 2). Cells carrying pVJ21 and pBAD-PH show very high β-galactosidase activities when arabinose is added, even at a low arabinose concentration that is not sufficient to activate the *toxT*::*lacZ* reporter construct by pBAD-PH alone (Table 2), suggesting a synergistic effect of ToxR/S and TcpP/H in activating the *toxT* promoter.

DISCUSSION

In the present study, we used a novel approach to select *V. cholerae* strains that are at least partially constitutive for production of TCP pili. We used the recently described phage derivative CTXΦ-Kan, which employs TCP as its receptor (10), to identify genes in *V. cholerae* that, when mutated, allow pili production during growth under nonpermissive environmental conditions (elevated pH or the presence of the drug procaine). After transduction, piliated cells become antibiotic resistant and can be selected from a large background of nonpiliated wild-type cells. In conjunction with the use of Tn*bla* (9) as the mutagenizing agent, this method allows the identification of membrane proteins that are involved in the regulation of pili production in response to environmental stimuli. The production of the type IV pilus, TCP, in *V. cholerae* is strongly influenced by growth conditions such as temperature, pH, and osmolarity (3), and several regulatory proteins have been identified that are involved in pilus expression (5). Our selection technique for mutants that constitutively express TCP has identified several known as well as previously unknown genes involved in TCP regulation.

Three mutant *V. cholerae* strains were characterized and the transposon insertions were mapped. One mutant strain had a Tn*bla* insertion in a gene homologous to *nqrB* from *V. alginolyticus*, a gene believed to encode a subunit of a Na^+-translocating NADH:ubiquinone oxidoreductase (17, 18). In *V. alginolyticus* this enzyme is induced under alkaline pH and produces an electrochemical gradient of sodium ions during aerobic respiration that is used to drive ATP synthesis, flagellar rotation, and solute transport. This alternative to H^+ coupling in membrane energetics allows the bacteria to maintain a neutral intracellular pH in an alkaline environment. The *V. cholerae nqrB* mutant strain showed much reduced motility compared with wild-type cells when assayed in soft agar (data not shown); however, it is not clear how this mutation is connected to TCP production. Nonmotile mutants of *V. cholerae* have previously been reported to be partially constitutive for ToxR-regulated gene expression (25).

Another transposon mutant was found to have an insertion in *tcpI*, whose product was previously implicated in the negative regulation of TCP production (19). *tcpI* was identified as a ToxR-activated gene that encodes an inner membrane protein with extensive sequence similarity to the highly conserved signaling domain in methyl-accepting membrane chemoreceptors (26). Interestingly, the *tcpI*::Tn*bla* mutant isolated in the present study produces pili even after growth at pH

Table 2. ToxR/S and TcpP/H activation of *toxT*

Strain	β-Galactosidase activity (Miller units) after growth in arabinose		
	0%	0.002%	0.02%
Δ*tcpP* Δ*toxR toxT*::*lacZ*	21.47 ± 3.95	26.16 ± 2.53	25.60 ± 2.15
Δ*tcpP* Δ*toxR toxT*::*lacZ* (pVJ21)	39.59 ± 4.87	55.11 ± 3.52	53.09 ± 2.53
Δ*tcpP* Δ*toxR toxT*::*lacZ* (PBAD-PH)	22.66 ± 6.95	20.32 ± 0.76	368.22 ± 31.38
Δ*tcpP* Δ*toxR toxT*::*lacZ* (pBAD-PH, pVJ21)	36.94 ± 2.56	770.66 ± 50.52	2,346.27 ± 429.23

β-Galactosidase activities are shown for a Δ*tcpP* Δ*toxR* double mutant *V. cholerae* strain carrying a *toxT*::*lacZ* reporter construct in the chromosome with plasmids expressing the *toxR* and *toxS* genes constitutively (pVJ21) and/or the *tcpP* and *tcpH* genes from an arabinose-inducible promoter (pBAD-PH). Results are mean ± SD.

8.5; however, as in the parental strain, pili production is still inhibited by elevated temperature and procaine (data not shown). This suggests that TcpI is mainly involved in the down-regulation of TCP in response to alkaline environments.

A third mutant carried a transposon insertion in *tcpP*. This gene has previously been thought to have some role in regulation, as it lies in an operon with *tcpH*, a known positive regulator of TCP. A Tn*phoA* insertion in *tcpH* resulted in cells with sporadic, but visually normal, pili (19). More recently, evidence suggested that spontaneous null mutations in *tcpH* produce little or no TCP or cholera toxin (27). Similarly, we expected a transposon insertion in *tcpP* to lead to reduced rather than deregulated pili production. Indeed, an in-frame *tcpP* deletion mutant strain did not produce TCP when assayed by phage transduction but was complemented for pili production by a plasmid carrying the *tcpP* gene. As the Tn*bla* insertion in this strain occurred in the very carboxyl-terminal region of TcpP, it was possible that the resulting fusion protein remains functional. In fact, the cloned *tcpP*::*bla* hybrid gene did complement a Δ*tcpP* strain, indicating that this TcpP-Bla fusion protein is active.

The Δ*tcpP* strain was complemented by a *toxT*-expressing plasmid, indicating that the mutation results in a defect in regulatory rather than structural aspects of pilus assemble. A BLAST search of TcpP against proteins in the data banks revealed homology of the amino-terminal region of TcpP to several regulatory proteins, including PsaE from *Y. pestis* and ToxR from *V. cholerae*. PsaE, in conjunction with PsaF, is involved in the regulation of pH 6 antigen, a fimbrial surface structure found on *Y. pestis* and *Yersinia pseudotuberculosis* (28) in response to changes in temperature and pH. A similar pilus structure is found in *Yersinia enterolytica*, called Myf, in which *myfE* and *myfF* appear to be homologues of *psaE* and *psaF*. PsaE, like ToxR of *V. cholerae*, is an integral inner membrane protein with a amino-terminal cytoplasmic region with sequence similarity to transcriptional regulators and a periplasmic carboxyl-terminal region. PsaF as well as MyfF resemble ToxS from *V. cholerae*, as they are predicted to be located mostly in the periplasm with the hydrophobic amino terminus being either inserted in the inner membrane or cleaved as a signal peptide (28, 29). TcpP and TcpH of *V. cholerae* appear to have structures similar to these pairs of transcription activators (Fig. 1). This led us to investigate whether TcpP alone or in conjunction with TcpH is a transcriptional activator. Indeed, when *tcpP* was expressed in *V. cholerae* it activated a chromosomal *toxT*::*lacZ* reporter construct. This activity was markedly enhanced by the simultaneous expression of *tcpH*. Additionally, *tcpP* as well as *tcpPH*, when strongly expressed from an independent promoter, complemented a *toxR*-deletion strain for phage transduction and for β-galactosidase activity of the *toxT*::*lacZ* reporter construct. It was recently shown that the promoter upstream of *tcpPH* is transcribed independently of ToxR and ToxT in classical *V. cholerae* strains and is regulated by growth conditions such as temperature and pH (27, 30). Furthermore, there is some rather weak evidence that indicates that TcpP is involved in the regulation of the *tcpP* and *tcpI* promoters (30, 31). We propose a model where both ToxR/S and TcpP/H are involved in sensing various environmental and internal stimuli and are required for the production of TCP in *V. cholerae* by cooperating in the activation of the *toxT* promoter. Both TcpP and ToxR appear to be homologous membrane regulatory proteins; similarly, although TcpH and ToxS are not homologous, their predicated topology suggests that they too reside within the inner membrane and have similar topologies (Fig. 1*C*). Thus, it is conceivable that our observed requirement for both TcpP/H and ToxR/S for optimal expression of the *toxT* gene may reflect the interaction of these membrane regulatory proteins with each other in a complex. Alternatively, these pairs of regulatory proteins may activate different promoters upstream of *toxT* in a manner that produces the observed cooperativity between TcpP/H and ToxR/S. The membrane localization of these four regulatory proteins also suggests their interaction with membrane components of the motility and chemotaxis systems as well as other membrane proteins involved in the energizing of the cytoplasmic membrane.

We thank Victor DiRita for the generous gift of the plasmid pRSI-2 and the Δ*toxT* strain, Karl Klose for kindly providing the Δ*toxR* strain and for the plasmids pBAD-R and pBAD-T, and Wei Lin for the plasmid pWL10. We also thank Eric Rubin for many helpful discussions and for critically reading this manuscript. We are grateful to John W. Tobias and Nicholas Judson for assistance with the graphic programs. This study was supported by National Institutes of Health Grant AI 18045-13.

1. Taylor, R. K., Miller, V. L., Furlong, D. B. & Mekalanos, J. J. (1987) *Proc. Natl. Acad. Sci. USA* **84,** 2833–2837.
2. Peterson, K. M. & Mekalanos, J. J. (1988) *Infect. Immun.* **56,** 2822–2829.
3. Miller, V. L. & Mekalanos, J. J. (1988) *J. Bacteriol.* **170,** 2575–2583.
4. Skorupski, K. & Taylor, R. K. (1997) *Proc. Natl. Acad. Sci. USA* **94,** 265–270.
5. DiRita, V. J. (1995) in *Two-component Signal Transduction*, eds. Hoch, J. A. & Silhavy, T. J. (Am. Soc. Microbiol., Washington, DC), pp. 351–365.
6. Miller, V. L., Taylor, R. K. & Mekalanos, J. J. (1987) *Cell* **48,** 271–279.
7. DiRita, V. J. & Mekalanos, J. J. (1991) *Cell* **64,** 29–37.
8. DiRita, V. J. (1992) *Mol. Microbiol.* **6,** 451–458.
9. Reidl, J. & Mekalanos, J. J. (1995) *Mol. Microbiol.* **18,** 685–701.
10. Waldor, M. K. & Mekalanos, J. J. (1996) *Science* **272,** 1910–1914.
11. Mekalanos, J. J., Swartz, D. J., Pearson, G. D., Harford, N., Groyne, F. & de Wilde, M. (1983) *Nature (London)* **306,** 551–557.
12. Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. E., Seidman, J. G., Smith, J. A. & Struhl, K. (1991) *Current Protocols in Molecular Biology* (Wiley, New York).
13. Donnenberg, M. S. & Kaper, J. B. (1991) *Infect. Immun.* **59,** 4310–4317.
14. Skorupski, K. & Taylor, R. K. (1996) *Gene* **169,** 47–52.
15. DiRita, V. J., Parsot, C., Jander, G. & Mekalanos, J. J. (1991) *Proc. Natl. Acad. Sci. USA* **88,** 5403–5407.
16. Miller, J. H. (1972) *Experiments in Molecular Genetics* (Cold Spring Harbor Lab. Press, Plainview, NY).
17. Beattie, P., Tan, K., Bourne, R. M., Leach, D., Rich, P. R. & Ward, F. B. (1994) *FEBS Lett.* **356,** 333–338.
18. Hayashi, M., Hirai, K. & Unemoto, T. (1995) *FEBS Lett.* **363,** 75–77.
19. Shaw, C., Peterson, K. M., Mekalanos, J. J. & Taylor, R. K. (1988) in *Advances in Research on Cholera and Related Diarrheas*, eds. Takeda, Y. & Sack, R. B. (KTK Science Publishers, Tokyo), pp. 5–17.
20. Lindler, L. E. & Tall, B. D. (1993) *Mol. Microbiol.* **8,** 311–324.
21. Bajaj, V., Hwang, C. & Lee, C. A. (1995) *Mol. Microbiol.* **18,** 715–727.
22. DiRita, V. J., Neely, M., Taylor, R. K. & Bruss, P. M. (1996) *Proc. Natl. Acad. Sci. USA* **93,** 7991–7995.
23. Higgins, D. E. & DiRita, V. J. (1994) *Mol. Microbiol.* **14,** 17–29.
24. Miller, V. L., DiRita, V. J. & Mekalanos, J. J. (1989) *J. Bacteriol.* **171,** 1288–1293.
25. Gardel, C. & Mekalanos, J. J. (1995) *Infect. Immun.* **64,** 2246–2255.
26. Harkey, C. W., Everiss, K. D. & Peterson, K. M. (1994) *Infect. Immun.* **62,** 2669–2678.
27. Carroll, P. A., Tashima, K. T., Rogers, M. B., DiRita, V. J. & Calderwood, S. B. (1997) *Mol. Microbiol.* **25,** 1099–1111.
28. Yang, Y. & Isberg, R. (1997) *Mol. Microbiol.* **24,** 499–510.
29. Iriarte, M. & Cornelis, G. R. (1995) *J. Bacteriol.* **177,** 738–744.
30. Thomas, S., Williams, S. G. & Manning, P. (1995) *Gene* **166,** 43–48.
31. Manning, P. (1997) *Gene* **192,** 63–70.