

Examination of Diverse Toxin-Coregulated Pilus-Positive *Vibrio cholerae* Strains Fails To Demonstrate Evidence for *Vibrio* Pathogenicity Island Phage

Shah M. Faruque,¹ Jun Zhu,² Asadulghani,¹ M. Kamruzzaman,¹
and John J. Mekalanos^{2*}

Molecular Genetics Laboratory, International Centre for Diarrhoeal Disease Research, Bangladesh, Dhaka-1212, Bangladesh,¹ and Department of Microbiology and Molecular Genetics, Harvard Medical School, Boston, Massachusetts 02115²

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The major virulence factors of toxigenic *Vibrio cholerae* are cholera toxin, which is encoded by a lysogenic filamentous bacteriophage (CTX Φ), and toxin-coregulated pilus (TCP), an essential colonization factor that is also the receptor for CTX Φ . The genes involved in the biosynthesis of TCP reside in a pathogenicity island, which has been reported to correspond to the genome of another filamentous phage (designated VPI Φ) and to encode functions necessary for the production of infectious VPI Φ particles. We examined 46 *V. cholerae* strains having diverse origins and carrying different genetic variants of the TCP island for the production of the VPI Φ and CTX Φ in different culture conditions, including induction of prophages with mitomycin C and UV irradiation. Although 9 of 10 *V. cholerae* O139 strains and 12 of 15 toxigenic El Tor strains tested produced extracellular CTX Φ , none of the 46 TCP-positive strains produced detectable VPI Φ in repeated assays, which detected as few as 10 particles of a control CTX phage per ml. These results contradict the previous report regarding VPI Φ -mediated horizontal transfer of the TCP genes and suggest that the TCP island is unable to support the production of phage particles. Further studies are necessary to understand the mechanism of horizontal transfer of the TCP island.

Cholera is a severe dehydrating diarrhea caused by toxigenic strains of the gram-negative bacterium *Vibrio cholerae*. The profuse watery diarrhea is mainly due to an enterotoxin (cholera toxin [CT]) produced by *V. cholerae* (10, 22) when it colonizes the small intestine. Colonization of the brush border of the small intestine is assumed to be mediated by a rigid pilus colonization factor designated the toxin-coregulated pilus (TCP), the expression of which is coordinately regulated with expression of cholera toxin (20, 45, 46).

In *V. cholerae*, the major virulence genes are in clusters. The *ctxAB* genes encoding cholera toxin reside in the genome of a lysogenic filamentous phage (CTX Φ) (47), whereas the genes encoding the major colonization factor, TCP, are encoded by the TCP gene cluster or island (7, 30, 34, 40, 43, 44). Since the genes of the TCP cluster are not present in many strains of *V. cholerae*, this region was clearly recently acquired by *V. cholerae* through horizontal gene transfer (30, 34, 43, 44).

The major subunit of TCP is encoded by the *tcpA* gene, but pilus formation and function require the products of several other genes located on the chromosome adjacent to the *tcpA* gene. Immediately adjacent to and downstream of the *tcp* cluster is the *acf* gene cluster (8), which is assumed to encode an accessory colonization factor, although the exact nature of this colonization factor is unclear. A putative integrase gene (*int*) is located downstream of and adjacent to the *tcp-acf* gene cluster (18, 30). A nearly 40-kb region, including the *tcp-acf* gene

cluster, is flanked on both sides by putative 20-bp *att*-like attachment sequences (Fig. 1) (26, 30). This structural feature, together with the cluster's association with pathogenic strains of *V. cholerae*, prompted Karaolis and colleagues to rename the region the *Vibrio* pathogenicity island (VPI) (26). The VPI is absent from most nontoxigenic environmental strains, but it has recently been found in a variety of environmental strains (26, 36–38, 44).

Remarkably, the cholera toxin-converting bacteriophage CTX Φ uses TCP as its receptor for infecting cells (47). Since CTX Φ uses TCP as its receptor, it appears that acquisition of the TCP island is the initial genetic element acquisition required for origination of epidemic strains. However, the mechanism of horizontal transfer of the TCP island is controversial (34, 43, 47). In a previous report the authors suggested that the TCP island corresponds to the genome of another filamentous phage, designated VPI Φ (25). However, this finding has not been verified independently and has not been supported by any other investigation. In the present study, we reexamined *V. cholerae* strains having diverse origins and carrying genetic variants of the TCP island for the production of VPI Φ . Our results do not support the conclusion that VPI Φ particles are produced by TCP island-positive *V. cholerae*.

MATERIALS AND METHODS

Bacterial strains. A total of 46 TCP island-positive *V. cholerae* strains were analyzed in the present study. These strains included 6 classical biotype strains and 18 El Tor biotype strains of *V. cholerae* O1, 10 strains of *V. cholerae* O139, and 12 *V. cholerae* strains belonging to six different non-O1, non-O139 serogroups. The clinical O1 and O139 strains were either obtained from our culture

* Corresponding author. Mailing address: Department of Microbiology and Molecular Genetics, Armenise I-421, Harvard Medical School, 200 Longwood Avenue, Boston, MA 02115. Phone: (617) 432-1935. Fax: (617) 738-7664. E-mail: jmekalanos@hms.harvard.edu.

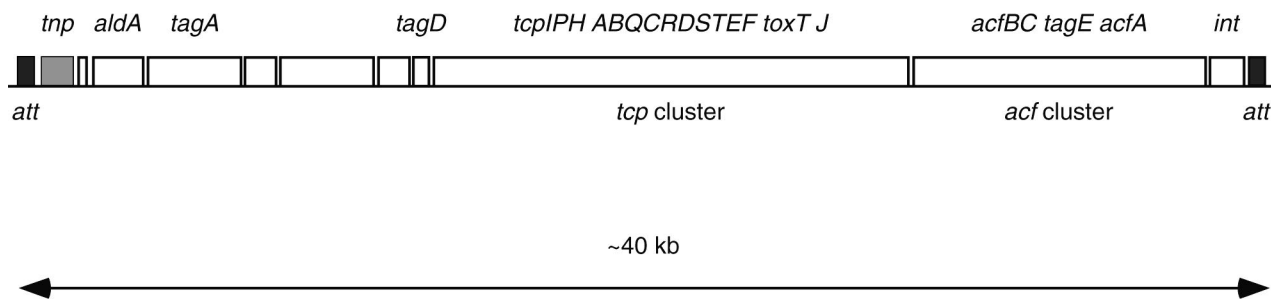


FIG. 1. Genetic organization of the TCP island of *V. cholerae*. att sites are at both ends, and the putative integrase gene is near the right end of the island. The transposase sequence is near the left end of the island (grey box). To improve clarity, the att sites are not drawn to scale.

collection or freshly isolated from diarrhea patients at the treatment center of the International Centre for Diarrheal Disease Research, Bangladesh (ICDDR,B) located in Dhaka, Bangladesh. Environmental isolates of O1 and O139 vibrios were isolated from surface water samples collected in Dhaka, whereas the non-O1, non-O139 strains were initially cultured from three different freshwater lakes and ponds in the eastern part of Calcutta, India (3). These strains carry genetic variants of the TCP island with new alleles of several TCP island genes, including *tcpA* and *toxT* (36). The strains analyzed in this study are described in Table 1. Strains were stored either in lyophilized form or in sealed deep nutrient agar at room temperature. Before use, the identities of the cultures were verified by biochemical and serological methods (48). All strains were also tested for the presence of the *ctx* and *tcp* gene clusters by using specific DNA probes or PCR assays as described below.

Induction of lysogens. *V. cholerae* strains were either treated with mitomycin C or subjected to UV irradiation to induce possible prophages carried by the strains. Strains were grown in Luria-Bertani broth (LB) at 30°C to an absorbance at 540 nm of 0.2. The cells were collected by centrifugation, washed, and resuspended in fresh LB. Each suspension was divided into aliquots to which mitomycin C (Sigma Chemical Company, St. Louis, Mo.) was added at a concentration of 20 ng/ml. To analyze induction by UV irradiation, 2-ml aliquots of

cultures containing 2×10^5 cells were spread on a UV-transparent tray, placed on a UV transilluminator (model T-2201; Sigma), and exposed for 2 to 3 min. The treated cells were washed with LB and inoculated into test flasks containing 500 ml of fresh LB. Different sets of cultures were grown at 30 and 37°C and for 6 and 16 h (overnight) with shaking. The culture supernatants were analyzed for extracellular phage particles as described below. Strains grown in a similar way but without mitomycin C or UV irradiation were also analyzed for phage production.

Isolation of phage DNA. Phage preparations were made by using 500-ml LB cultures and the method described by Karaolis et al. (25), and total nucleic acids were extracted from these preparations. In brief, cultures were centrifuged at $10,000 \times g$, and the supernatants were sterilized by filtration. To confirm that the filtrates from the culture supernatants did not contain bacterial cells, aliquots of the filtrates were streaked on Luria agar plates and incubated overnight at 37°C. Pancreatic DNase I (100 U/ml) and RNase A (50 µg/ml) were added to the filtrates and incubated at room temperature for 3 h. NaCl and polyethylene glycol 8000 were added to final concentrations of 1 M and 10% (wt/vol), respectively, and the mixtures were left to precipitate overnight at 4°C. The supernatants were centrifuged at $11,000 \times g$ for 20 min, and the pellets were resuspended in 2 ml of SM buffer. Each solution was extracted twice with an equal volume of

TABLE 1. Analysis of 46 strains of TCP-positive *V. cholerae* belonging to different serogroups for production of CTXΦ and VPIΦ particles

Strain(s)	No. of strains analyzed	Serogroup and/or biotype	Presence of genes							Production of phages in supernatant ^b		
			<i>tcpA</i> ^a	<i>tcpI</i>	<i>toxT</i> ^a	<i>acfB</i>	<i>rsfR</i>	<i>ctxA</i>	<i>zot</i>	VPI _φ	CTX _φ ^c	
Clinical isolates												
S-224, S-262, L-351, L-262, O395, AE-2882	6	O1 classical	C	+	+	+	+	+	+	+	-	-
SA317, SA406, SA508	3	O1 El Tor	E	+	+	+	-	-	-	-	-	-
G-755, N-16961, P-27457, AM-13860, AM-11726, AF-1471, AP-13547, AP-13530, AM-11706	9	O1 El Tor	E	+	+	+	+	+	+	+	-	+
P-28108, P-27821, AL-12890	3	O1 El Tor	E	+	+	+	+	+	+	+	-	-
AI-1836	1	O139	E	+	+	+	+	+	+	+	-	-
AL-11089, AP-32541, AK-17334, AR-1432, AR-1468, AL-12077	6	O139	E	+	+	+	+	+	+	+	-	+
Surface water isolates												
EV-02, EV-11, 102V138	3	O1 El Tor	E	+	+	+	+	+	+	+	-	+
SB-Lake, 224V541, Env-91	3	O139	E	+	+	+	+	+	+	+	-	+
SCE4, SCE359	2	O8	C	+	+	+	-	-	-	-	-	-
SCE223, SCE354	2	O27	Env	+	+	+	+	+	+	+	-	-
SCE225, SCE226, SCE227	3	O35	C	+	Env	+	+	-	-	-	-	-
SCE256, SCE264	2	O42	C	+	Env	+	+	-	-	-	-	-
SCE263	1	O10	C	+	Env	+	+	+	-	-	-	-
SCE340, SCE341	2	O69	E	+	Env	-	-	-	-	-	-	-

^a Env, +, present; -, not present; new alleles of the *tcpA* or *toxT* genes, which are different from those normally found in epidemic strains; C, classical *tcpA* type gene present; E, El Tor type *tcpA* gene present.

^b Most strains carried other unidentified phage genomes, as shown by analysis of the total nucleic acids extracted from phage preparations of the strains, but these nucleic acids did not hybridize with probes for CTXΦ or VPIΦ. Strains were grown in the presence of mitomycin C (20 ng/ml) for phage induction (see text for details).

^c CTXΦ was produced irrespective of whether strains were grown at 30 or 37°C.

phenol-chloroform and then once with chloroform to disrupt possible phage particles, and the total nucleic acid was precipitated with ethanol. The pellet was washed with 80% ethanol, vacuum dried, and resuspended in a final volume of 50 μ l. Preparations containing phage DNA were identified by PCR and Southern blot hybridization. For each PCR assay, 2 to 5 μ l of a solution was used, whereas 10 μ l was used for Southern blot analysis. Serial dilutions in LB of CTX-Km Φ (10 to 10⁴ particles/ml) isolated from a mitomycin C-induced culture of strain SM44 (10) were used as positive controls for the phage assay. CTX-Km Φ derived from SM44 was quantified as described previously by incubating serially diluted filter-sterilized culture supernatants with strain RV508 and then determining the number of Km^r colonies (11, 47).

Probes and PCR assays. The gene probes used in this study to analyze different *V. cholerae* strains included a 0.5-kb *Eco*RI fragment of pCVD27 encoding part of the *ctxA* gene (23) and an 840-bp region internal to the *zot* gene, amplified by PCR from the recombinant plasmid pBB241 as described previously (14). The El Tor and classical biotype-specific *rstR* probes were *Sac*I-*Xba*I fragments of pHK1 and pHK2, respectively (28). PCR assays specific for the *tcpA*, *tcpI*, *acfB*, and *toxT* genes of the TCP pathogenicity island, as well as the *ctxA* and *zot* genes of CTX prophage, have been described previously (3, 9, 14, 15, 27). Phage nucleic acid preparations were tested both by PCR assays and by Southern blot hybridization with specific probes. The multiplex PCR assay for classical or El Tor biotype-specific *tcpA* genes (27) was used to detect the presence of possible VPI Φ genomes. To detect possible VPI Φ produced by environmental non-O1, non-O139 strains carrying the *env* variant of *tcpA*, a PCR assay with primers flanking *tcpA* (*tcp*I-F [5'GCCGTCTCCGCATTAAGCTCTGCAC] and *tcp*Q-R [5'GAGGACTGTTCTGCAATCTGCTCAT]) was performed as described previously (36). The 5.4-kb PCR-generated amplicon corresponding to the *tcpI*-*Q* region and the *tcpA* amplicon were also used as probes to screen for possible VPI Φ by Southern blot hybridization of phage nucleic acids. For PCR-based detection of the CTX Φ genome the *ctxA* PCR was used, whereas for detection of the CTX-Km Φ genome a PCR assay for the *zot* gene was used (14). A strand-specific oligonucleotide probe (5'TCTATCTCTGTAGCCCCTATTACG) was also used to specifically identify the (+) strand of the single-stranded genome of CTX Φ .

Colony or Southern blots were prepared by using nylon filters (Hybond; Amersham Biosciences, Uppsala, Sweden) and were processed by standard methods (33). The polynucleotide probes were labeled by random priming (16) by using a random primer DNA labeling kit (Bethesda Research Laboratories, Gaithersburg, Md.) and [α -³²P]dCTP (3,000 Ci/mmol; Amersham). Oligonucleotide probes were labeled by 3' tailing by using terminal deoxynucleotidyl transferase and [α -³²P]dCTP (Amersham). Southern blots and colony blots were hybridized with the labeled probes and autoradiographed as described previously (11).

Construction of Km^r-*oriR6K* insertions in the TCP island. Internal fragments of *tcpI* (VC0825), *int* (VC0847; encodes a putative integrase), *tnp* (VC0817; encodes a putative transposase), and *aldA* (VC0819; encodes an aldehyde dehydrogenase) were cloned into pNJ16, which contains a Km^r gene and an origin of replication (*oriR6K*) that is not functional in *Vibrio* (21). The resulting plasmids were inserted into the chromosome of El Tor strain N16961 or classical strain O395 by homologous recombination.

Infant mouse infections. Infant mouse colonization assays were performed as previously described (17). In brief, 6-day-old infant CD-1 mice were inoculated intragastrically with a mixture of overnight cultures of N16961 *tcpI*::Km^r-*oriR6K* (Km^r Sm^r) and Vc857 (Nal^r TCP⁻) at a ratio of approximately 1:5. After 24 h of incubation, the mice were sacrificed, and the small intestines were removed and homogenized. The homogenates were plated on Luria agar with appropriate antibiotics to determine the number of kanamycin-resistant N16961 or Vc857 bacteria present.

RESULTS AND DISCUSSION

The only previous study (25) to demonstrate production of VPI Φ and transfer of the TCP island to a recipient strain raised a number of intriguing questions regarding the biology and ecology of VPI Φ (31). However, there has been no other confirmation of the existence of VPI Φ , despite the important implications of the discovery of VPI Φ for understanding the role of vibriophages in the emergence and evolution of epidemic *V. cholerae* strains. We therefore decided to reexamine

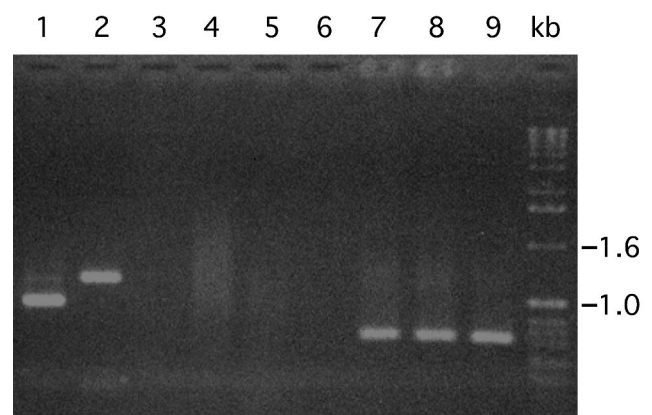


FIG. 2. PCR analysis of phage nucleic acid preparations of *V. cholerae* strains carrying the TCP island. Preparations from various strains were tested by PCR for *tcpA* (lanes 4 to 6) and *ctxA* (lanes 7 to 9). The following *V. cholerae* strains were tested: lanes 4 and 7, AL-11089; lanes 5 and 8, AL-12077; lanes 6 and 9, P-27457. As positive controls, lanes 1 and 2 contained PCR assay mixtures from assays for *tcpA* in which chromosomal DNA templates from El Tor strain G-755 and classical strain 569B, respectively, were used. Lane 3 contained the *tcpA* PCR product of the chromosomal DNA template from strain Env-37, which lacks the *tcpA* gene. The numbers on the right indicate the positions of bands derived from a 1-kb DNA ladder (Invitrogen) used as molecular size markers.

the ability of diverse *V. cholerae* strains to produce VPI Φ by using the same culture conditions that were used in the original report describing VPI Φ (25). Of the 46 *V. cholerae* strains analyzed in this study, 44 were positive in PCR assays for the *tcpA*, *tcpI*, *toxT*, and *acfB* genes, indicating that these genes and presumably the entire TCP island are present in these strains (Table 1). Two strains were negative for the *acfB* gene but were positive for the *tcpA*, *tcpI*, and *toxT* genes. Except for three El Tor biotype strains and nine non-O1, non-O139 strains, all other strains were also positive for the CTX prophage, as shown by the presence of the *ctxA* and *zot* genes.

PCR analysis of total nucleic acid extracted from phage preparations indicated that all phage preparations were negative for the *tcpA* gene. Southern blot analysis of phage nucleic acid with probes for different TCP island genes was also negative (data not shown). The same nucleic acid extracts were tested for the CTX Φ -specific gene *ctxA* by using both PCR assays and DNA probe assays; representative data obtained in these experiments are presented in Fig. 2 and 3. Phage preparations from all toxigenic classical biotype strains were negative for the CTX Φ genome, while phage preparations from 12 of 15 toxigenic El Tor strains and 9 of 10 O139 strains were positive for CTX Φ production. These findings are consistent with previous studies, which showed that the classical biotype strains of *V. cholerae* O1 do not produce infectious CTX Φ , which has been attributed to a dysfunctional CTX prophage (6). It should be emphasized that the *ctxA* hybridizing sequences or the PCR templates that gave positive PCR results for *ctxA* were evidently present in phage particles, since these DNAs should have been the only DNAs protected from nuclease digestion during phage preparation. The same preparations did not give positive reactions in the PCR assay for *tcpA*. Thus, in the present study, the presence of CTX Φ and the

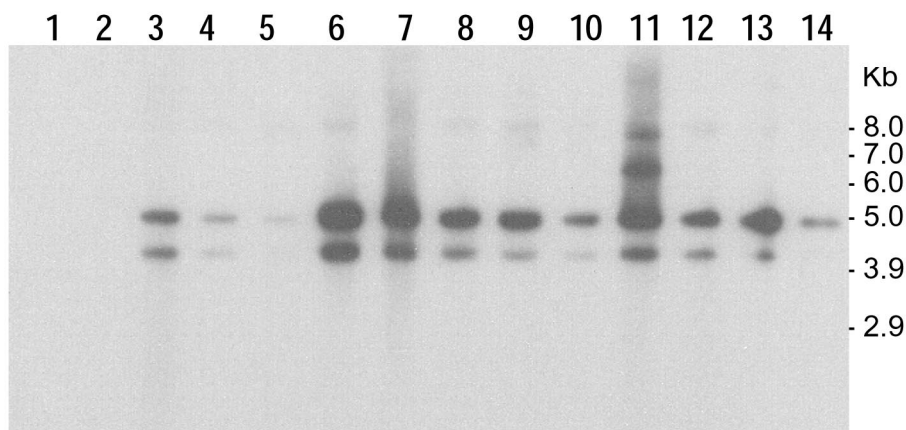


FIG. 3. Southern blot hybridization analysis of total phage nucleic acids derived from different *V. cholerae* strains by using a *ctxA* gene probe. The *V. cholerae* strains used for phage preparation were AL-12890, P-28108, AM-11726, AM-11706, EV-02, AL-11089, AL-12077, P-27457, N-16961, AP-32541, AF-1471, AR-1402, AR-1432, and G-755 (lanes 1 through 14, respectively). The numbers on the right indicate the positions of bands derived from a supercoiled DNA ladder (Invitrogen) used as molecular size markers.

absence of VPI Φ served as reciprocal controls and ruled out the possibility of chromosomal DNA contamination of the phage nucleic acid preparations.

In the case of LB mixed with exogenous CTX-Km Φ and used as a positive control, a minimum of 5×10^4 phage particles added to 500 ml of medium (10^2 particles/ml) could be distinctly detected by Southern blot hybridization of 10 μ l of the ultimate phage nucleic acid preparation by using a *zot* probe. Positive PCR results were obtained with less than 1 μ l of the phage nucleic acid preparation (data not shown), which is theoretically equivalent to detecting the phage in a solution containing fewer than 10 particles/ml.

DNA-damaging agents, such as mitomycin C and UV irradiation, are known to induce lysogenic phages. We therefore used these agents to encourage phage production. Despite treatment with either mitomycin C or UV irradiation, none of the strains produced VPI Φ . The production of CTX Φ , however, was markedly influenced by both mitomycin C and UV irradiation. In the absence of any induction, only 5 strains produced CTX Φ , but with induction 21 of 25 toxigenic O1 El Tor and O139 strains produced extracellular CTX Φ particles. In view of the report that hemagglutinin protease produced during the late log phase of a culture can inactivate CTX Φ (29), in the present study we included precautions to ensure that any VPI Φ present in the culture supernatants was not degraded by proteases (thus escaping detection). We therefore tested culture supernatants for the presence of phage after 6 h of growth, as well as after 16 h of growth. It should be noted that the presence of CTX Φ served as an indicator, and since CTX Φ was clearly detected in cultures grown under either of these conditions, it is highly unlikely that VPI Φ was produced and degraded by extracellular proteases produced by the bacteria.

Another method for detecting phage is to monitor the transfer of genetic material by transduction. This method is commonly used for detecting and quantifying CTX Φ transfer (47). We therefore constructed several Km^r-marked versions of the TCP island and examined their ability to move into *Escherichia coli* or TCP-negative *V. cholerae* strains. Km^r-oriR6K was in-

serted into *tcpI*, *aldA*, *tnp*, and *int* of N16961 and O395, and the resulting strains were used as donors for transduction of *E. coli* DH5 α pir or Vc857, a *V. cholerae* environmental isolate that lacks the entire TCP island. No TCP island transfer was detected by standard conjugation and transduction techniques (data not shown). We repeated these assays under various conditions, such as *toxT* overproduction (which induces expression of most TCP genes), overproduction of the putative phage integrase, starvation, or conditions favoring biofilm formation. Again, not a single TCP transfer event was detected.

It has been reported that the efficiency of CTX Φ transduction in El Tor strains is greatly increased during host infection (47). We therefore coinoculated N16961 *tcpI*::Km and the recipient strain Vc857 into 6-day-old infant mice and recovered bacteria after 24 h of incubation. For all six mice examined, we did not recover a single Km^r Vc857 bacterium, while an average of 2.4×10^6 N16961 *tcpI*::Km bacteria were recovered per mouse. Therefore, we concluded that TCP islands are not transferred between bacteria under such in vivo conditions.

The putative VPI Φ is presumed to have a TCP-like morphology and to be composed of TcpA subunits, but expression of TCP is known to vary under different growth conditions (35). We therefore used two different temperatures (30 and 37°C) to examine the production of VPI Φ . Karaolis et al. observed VPI Φ production at 30°C in the classical biotype strain O395 and at 37°C in the El Tor biotype strain N16961 (25). We included both these strains in our study, but in our assays neither of these strains was positive for the production of VPI Φ , although strain N16961 produced CTX Φ under all conditions tested (Table 1).

Karaolis et al. reported that classical strain O395 produced VPI Φ , as shown by PCR detection of VPI DNA within phage particles that were produced at 30°C and that were immunoprecipitable with anti-TCP antibody (25). However, these particles appeared to be morphologically identical to the TCP bundles that are known to be produced by O395 (45). TCP production in O395 is tightly linked to autoagglutination (4, 45). Phage preparations that we derived from highly autoagglutinated cultures of O395 grown at 30°C consistently failed to

TABLE 2. Transduction efficiency of phage K139 and the TCP island

Supernatant ^a	Transduction efficiency with the following recipients:			
	RV79 (TCP ⁺)		Vc857 (TCP ⁻) ^b	
	Ap ^r	Km ^r	Ap ^r	Km ^r
N16961ΩK139 (<i>glo::bla</i>), <i>tcp::Km^c</i>	5×10^{-1}	$<1 \times 10^{-6d}$	5×10^{-4}	$<1 \times 10^{-6d}$
O395ΩK139 (<i>glo::bla</i>), <i>tcp::Km^c</i>	6×10^{-1}	$<1 \times 10^{-6d}$	5×10^{-4}	$<1 \times 10^{-6d}$

^a There was 10^6 PFU of phage K139 per ml in each supernatant.

^b A number of TCP⁻ strains were tested as recipients.

^c Constructs with Km^r-*oriR6K* inserted in *tcpI*, *aldA*, *tnp*, and *int* were tested.

^d No Km^r colonies were found in 10^9 recipient cells.

show the presence of VPI DNA, as assessed by PCR or Southern blotting, nor did these phage preparations transduce the TCP island. We therefore concluded that the TCP made by O395 do not correspond to VPI phage particles.

In El Tor strains like N16961, TCP production occurs at a low level in vitro, but it can be detected by CTXΦ transduction either in vitro under the conditions described here or in vivo (13, 47). This supports the hypothesis that TCP were indeed expressed by the El Tor strains but that they also did not correspond to VPIΦ as assessed by PCR, Southern blotting, or (in the case of N16961) the ability to transduce the TCP island.

Since our marked TCP islands carried the *aphA-3* gene at locations different from that used by Karaolis et al. (25), there is a formal possibility that our insertions might have inactivated the phage. However, since our PCR and hybridization analyses of phage DNA preparations from unmarked wild-type strains did not detect VPIΦ either, we believe that our inability to detect VPIΦ or to mobilize the TCP island was not due to insertional inactivation of phage function.

Many *V. cholerae* strains are also lysogenized with a phage designated phage K139 (42). To determine whether this phage can facilitate transduction of the TCP island, we constructed donor strains containing K139 phage (marked with Ap^r in the *glo* gene) and Km^r-*oriR6K* inserted at various locations within the *tcp* region and used them as donor strains. K139 phage were transferred at high frequencies to recipients RV79 and Vc857 (10^{-1} and 10^{-4} , respectively), while TCP markers were not transferred at all (Table 2). Since it is possible that the recipients that we tested were not able to receive the TCP island, we also examined phage preparations directly by PCR. As shown in Fig. 4, the phage K139 gene *glo* was readily detected in the phage preparation, while the *tcpJ* gene was not. VC0815, which lies adjacent to the *tcp* genes but still within the TCP island, also was not detected in phage preparations. These data further indicated that under these conditions the TCP island is not transferred and is not detectable in a phage preparation.

A recent study found that genetic variants of the TCP island were carried by a number of environmental *V. cholerae* strains belonging to nonepidemic serogroups (1, 24, 36–38). Since environmental strains of *V. cholerae* are assumed to have evolved into pathogenic clones by acquisition of virulence genes, the TCP gene clusters in these environmental strains could be progenitors of the TCP island found in pathogenic clones and therefore genetically more closely related to an ancestral TCP phage that might have invaded a TCP-negative strain and converted it to a TCP-positive strain. Using this

assumption, we tested environmental non-O1, non-O139 strains for production of VPIΦ. However, analysis of 12 strains, including 2 strains carrying a new allele of the *tcpA* gene, 8 strains carrying a new allele of the *toxT* gene, and 2 strains carrying conventional *tcpA* and *toxT* genes, showed that none of these strains produced VPIΦ (Table 1).

In summary, although CTXΦ is produced by most toxigenic strains, none of the TCP island-positive strains analyzed here produced the putative VPIΦ. The detection limit of the PCR assays was less than 10 phage particles/ml. Assuming that 1 ml of an overnight culture contained $\sim 10^9$ bacterial cells, the phage would have been detectable even if 1 phage particle was produced per 10^8 cells. Since Karaolis et al. reported that VPIΦ donor strains produced more than 10^4 transducing particles per 3×10^7 donor cells, we estimated that our level of sensitivity was more than 10^6 times that needed to detect VPIΦ. Therefore, the results of the present study contradict the previous report demonstrating the production of VPIΦ and emphasize that further studies are needed to understand the mechanism involved in horizontal transfer of the TCP island.

The mechanism that significantly limits distribution of TCP genes to mostly strains of the O1 and O139 serogroups out of the more than 200 serogroups of *V. cholerae* should also be investigated. In a recent study, O'Shea and Boyd (41) demonstrated that the VPI was transferred to a recipient strain by the generalized transducing phage CP-T1 (19, 39). Since these authors observed transfer of the VPI only at low frequencies (10^{-8} to 10^{-9} per PFU), we concluded that the mechanism is simply generalized transduction of a chromosomal marker

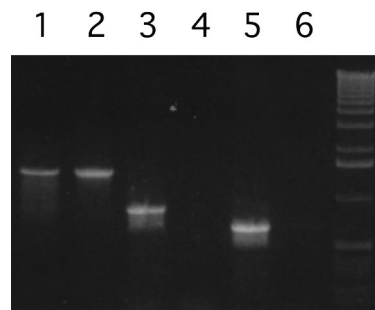


FIG. 4. PCR detection of genes in K139 phage and genes in and adjacent to the TCP island. Whole cells were used as templates in lanes 1, 3, and 5, while phage preparations were used in lanes 2, 4, and 6. Primers for the K139 gene *glo* were used in lanes 1 and 2; primers for *tcpJ* were used in lanes 3 and 4; and primers for VC0815 were used in lanes 5 and 6.

rather than a specific, VPI-mediated process. Therefore, we favor the hypothesis that the VPI corresponds to a satellite element similar to TSST element (32), P4 (2), or RS1 (5, 12). Such elements can be packaged efficiently into phage particles by a helper phage but are not viable phages themselves. We are currently exploring this hypothesis by isolating diverse vibriophages and testing them for efficient packaging and transfer of the VPI.

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