INFECTION AND IMMUNITY, Aug. 2002, p. 4735–4742 Vol. 70, No. 8
0019-9567/02/$04.00+0 DOI: 10.1128/IAI.70.8.4735–4742.2002

Vibrio Pathogenicity Island and Cholera Toxin Genetic Element-Associated Virulence Genes and Their Expression in Non-O1 Non-O139 Strains of *Vibrio cholerae*

Amit Sarkar,[1] Ranjan K. Nandy,[2] G. Balakrish Nair,[2] and Asoke C. Ghose[1]*

Department of Microbiology, Bose Institute, Calcutta-700 054,[1] and National Institute of Cholera and Enteric Diseases, Calcutta-700 010,[2] India

Received 9 October 2001/Returned for modification 4 March 2002/Accepted 9 May 2002

A non-O1 non-O139 *Vibrio cholerae* strain, 10259, belonging to the serogroup O53 was shown to harbor genes related to the vibrio pathogenicity island (VPI) and a cholera toxin (CT) genetic element called CTX. While the nucleotide sequence of the strain 10259 *tcpA* gene differed significantly (26 and 28%) from those of O1 classical and El Tor biotype strains, respectively, partial sequence analysis data of certain other VPI-associated genes (*aldA*, *tagA*, *tcpP*/*H*, *toxT*, *acfB*/*C*, and *int*) and intergenic regions (*tcpF* to *toxT* and *tcpH* to *tcpA*) of the strain showed only minor variations (0.4 to 4.8%) from corresponding sequences in O1 strains. Strain 10259 also contained CTX element-associated toxin genes with sequences almost identical to those of O1 strains. Growth of the organism in Luria broth (LB) under ToxR inducing conditions (30°C and pH 6.5) led to transcriptional activation of *tcpP*/*H*, *toxR*, *toxT*, and *tcpA* genes, but not of *ctxA*, as determined by reverse transcription-PCR (RT-PCR). Subsequent analysis revealed that strain 10259 possessed only two copies (instead of three or more copies found in epidemic-causing O1 or O139 strains) of the heptanucleotide (TTTTGAT) repeats in the intergenic region upstream of *ctxAB*. Therefore, a strain 10259 mutant was generated by replacement of this region with a homologous region (1.4 kb) derived from a *V. cholerae* O1 classical biotype strain (O395) that contained seven such repeats. The resultant recombinant strain (10259R) was found to be capable of coordinately regulated expression of *toxT*, *ctxA*, and *tcpA* when grown under the ToxR inducing conditions. Serological studies also demonstrated that the recombinant strain produced TcpA and a significantly (~1,000-fold) higher level of CT in vitro compared to that of the parent strain. Virulence gene expression in two other non-O1 non-O139 strains (serogroup O37) containing VPI and the CTX element was studied by RT-PCR and serological assay. One strain (S7, which was involved in an epidemic in Sudan in 1968) showed coordinately regulated expression of virulence genes leading to the production of both CT and TcpA in LB medium. However, the other strain, V2, produced RT-PCR-detectable transcripts of *toxT*, *ctxA*, or *tcpA* genes in the early phase (6 h), but not in the late phase (16 h) of growth in LB medium. These results are consistent with the low levels of production of CT and TcpA by the strain that were serologically detectable. The significance of these results is discussed in relation to the role of virulence genes and their expression to the pathogenic potential of *V. cholerae* strains belonging to non-O1 serogroups.

In humans, the disease cholera is caused by strains of the gram-negative bacterium *Vibrio cholerae* that belong to the O1 or O139 serogroup. The organism enters into the host during ingestion of contaminated water or food material, colonizes the small intestine, and produces an enterotoxin (cholera toxin [CT]) that is primarily responsible for the induction of massive loss of salt and water in the form of diarrhea (18). Colonization of the gut is facilitated through the expression of bundle-forming pilus structures (toxin-coregulated pilus [TCP]) on the surface of the bacterium (48). The expression of both CT and TCP is coordinately regulated at the transcriptional level by a cascade of signaling pathways that involve several transmembrane and cytosolic regulatory proteins (23, 45). Briefly, in response to appropriate environmental stimuli, the transmembrane protein ToxR, in association with ToxS of the *toxRS* operon, activates *toxT*, the gene encoding the cytosolic protein ToxT, which in turn activates transcription of genes for CT (*ctxAB*), the major structural protein subunit gene *tcpA*, and several others involved in TCP biosynthesis and secretion (23, 45, 54). More recently, membrane-associated proteins (e.g., TcpP and TcpH of the *tcpPH* operon) have been shown to play an additional regulatory role in the transcriptional activation of *toxT* (6, 15). Furthermore, activation of *tcpPH* is controlled, either positively (46) or negatively (2), by several regulatory elements. The *ctxAB* operon is located within a larger genetic element called CTX, which also encodes genes for several other toxins and accessory virulence factors (38). CTX was likely to be integrated into the host chromosome following lysogenic conversion of a filamentous bacteriophage, CTXϕ (51). Similarly, the *tcp* gene cluster has been shown to be a part of a 39-kb DNA region, referred to as a vibrio pathogenicity island (VPI), which contains a gene for the ToxT regulatory protein as well as several other clusters of genes of known and unknown function (19). Like CTX, the VPI has been proposed to be of another lysogenic bacteriophage origin (20). The ac-

* Corresponding author. Mailing address: Department of Microbiology, Bose Institute, P-1/12 CIT Scheme VIIM, Calcutta-700 054, India. Phone: 033-337-9416/9544. Fax: 91-33-334-3886. E-mail: acghosh@boseinst.ernet.in.

quisition of VPI by *V. cholerae* endows the organism with the ability to express TCP, which acts as a receptor for CTXϕ (20). The VPI and CTX genetic elements are primarily found in *V. cholerae* strains of O1 and O139 serogroups, which are associated with epidemic cholera (18, 19). The majority of strains belonging to about 200 other non-O1 non-O139 serogroups (53) do not contain genes for CT and/or TCP (18, 31, 47), although the gene for ToxR is ubiquitously present in these strains (34). The non-O1 non-O139 strains, which are predominantly isolated from an aquatic environment, are largely nonpathogenic in nature, although some of these are known to cause sporadic cases or occasional outbreaks of diarrhea in humans (30). Recently, however, *ctxAB*- and *tcp*-related genes have been shown to be present in certain strains of non-O1 non-O139 *V. cholerae* of both clinical and environmental origins (7, 14, 35, 36, 40, 42). This has raised important issues related to their evolution as well as relevance from the public health point of view. The point assumes considerable significance in view of the fact that strains belonging to non-O1 non-O139 serogroups have recently been implicated as the causative agents of a large number of cases of diarrhea in various parts of the world (1, 9, 41, 43). Evidently, documentation of the mere presence of the virulence-associated genes is not likely to provide sufficient information on the pathogenic potential of these strains, which is likely to depend on the presence of complex signaling pathways to couple appropriate environmental signals to virulence gene expression. Although some of the non-O1 non-O139 strains described earlier were shown to express detectable amounts of CT and/or TcpA protein in vitro (10, 14, 31, 32, 35), only limited information about their VPI and CTX is available so far (7, 14, 20), and no data on the regulation of virulence gene expression are available. In an earlier study, we characterized a new type of TcpA protein in a toxigenic *V. cholerae* strain, 10259, belonging to serogroup O53 (35). In the present communication, we describe partial characterization of VPI and CTX genetic elements of strain 10259 and provide information on its pathogenic potential by determining its ability to express virulence genes when grown in vitro under conditions that favor the expression of the same genes in *V. cholerae* O1 strains. We have extended this study by including data obtained with two other non-O1 non-O139 strains harboring VPI and CTX-related genes.

The bacterial strains and plasmids used in this study are listed in Table 1. The presence of virulence-associated genes in *V. cholerae* strains was determined by PCR amplification experiments with the primers listed in Table 2. All of these target genes, except *toxR*, are located in the VPI or CTX genetic element of the *V. cholerae* chromosome. Bacterial cells were grown overnight in Luria broth (LB) at 37°C, and chromosomal DNA was isolated from the harvested bacteria by a standard protocol. PCR amplification of target DNA was carried out in a thermal cycler (Perkin-Elmer) by essentially following the methodology described earlier (34). The reaction mixture was subjected to 30 cycles of amplification. Each cycle consisted of three successive steps in the following order: denaturation at 94°C for 30 s, annealing at 55°C for 50 s, and extension at 72°C for 50 s.

Our earlier study (34) demonstrated that the *toxR* gene was present in about 98% of *V. cholerae* strains tested, and strain 10259 was found to be no exception to this rule. The *toxR* gene

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Serogroup or characteristic[a]	Source or reference
Strains		
V. cholerae		
O395	O1 (classical/Ogawa), Sm^r	25
AD48	O1 (E1 Tor/Ogawa)	14
Co900	O1 (E1 Tor/Inaba)	Clinical isolate (local)
V1228	O1 (E1 Tor/Ogawa), Δ*tcpA*	49
10259	O53	10
V2	O37	10
S7	O37	3
E. coli SM10λ *pir*	*thi thr leu tonA lacA supE recA*::RP4-Tc::Muλ *pir* R6K	29
Plasmid		
pCVD 442	*sacB* gene containing suicide vector, Ap^r	12

[a] Sm^r, streptomycin resistant; Ap^r, ampicillin resistant.

nucleotide sequence of 10259 differed at only 3 base positions and 1 base position, respectively, from those of classical and El Tor strains (data not shown). Furthermore, PCR amplification of VPI-associated genes in the non-O1 non-O139 *V. cholerae* strain 10259 produced amplicons with all of the combinations of primers as shown in Fig. 1. Amplicon sizes were verified by comparison of the products with those generated with the *V. cholerae* O1 strain (O395). Some of the amplicons generated with strain 10259 were purified and sequenced with an automated DNA sequencer (Applied Biosystems) by using the end or internal primers. Partial nucleotide sequences of the genes *aldA*, *tagA*, *tcpP*/*H*, *tcpA*, *toxT*, *acfB*/*C*, and *int* of strain 10259 were determined and compared with those already available in the literature for *V. cholerae* O1 strains of both the classical and El Tor biotypes (GenBank accession no. X64098 and X74730 and AE004168 and AE004169, respectively). The summarized results presented in Table 3 show only minor variations (0.3 to 2.8%) between the 10259 sequence and the corresponding sequences from either classical or El Tor strains. In contrast, the nucleotide sequence of 10259 *tcpA* differed from those of classical and El Tor *tcpA* genes by 26 and 28%, respectively (35). Partial sequence analysis of the intergenic region between *tcpF-toxT* of 10259 revealed only minor (1%) variation from sequence of the corresponding region of *V. cholerae* O1 strains. However, the *tcpH-tcpA* intergenic sequence covering the promoter proximal region (302 bp) of *tcpA* showed 3.3 and 4.3% changes with the corresponding region sequences of classical and El Tor strains, respectively. No difference, however, was detectable at −10 and −35 regions upstream of *tcpA*. The GC content of the partially sequenced region of 10259 VPI was found to be 34.6%, which is quite comparable to those (35.5%) of VPIs of O1 strains (19), but differs from the overall GC content (47%) of the *V. cholerae* genome (16). It is therefore reasonable to conclude that, as is the case with *V. cholerae* O1, the 10259 VPI was also acquired from an outside donor. Generation of amplicons with

TABLE 2. List of primers used in this study

Primer[a]	Primer sequence (5′→3′)	Reference
VPI		
aldA-(F)	TGTGTTTCTCGTCCAATGCC	This study
aldA-(R)	TCGTCTTCGCAAGATGTCGAACTTGC	
tagA-(F)	GGTGGTAAGATATTCACTC	33
tagA-(R)	GAGACATCTATAGAATACTGGCTG	
tcpP-(F)	ACTCTGTGAATATCATCCTGCC	This study
tcpH-(R)	CTGGGTAAGCCAAACATTGG	
tcpI-(F)	GCCGTCTCCGCATTAAGCTCTGCAC	33
tcpA-(F)	CACGATAAGAAAACCGGTCAAGAG	33
tcpA-(R)	ACCAAATGCACGCCGAATGGAGC	
tcpQ-(R)	GAGGACTGTTCTGCAATCTGCTCAT	This study
toxT-(F)	ACTGTATAGCAAAGCATATTCAGAGA	This study
toxT-(R)	CGCGGATCCATACAATCGAAAATAGGA	
tcpF-(F)	CTGTCAAACCATATCAGC	This study
tcpJ-(R)	TAAAGTAAAGCCCGAGC	
acfB-F	AATGTCAGACTTTGGCG	This study
acfC-R	CGAGATCGATAAGTCTTCC	
orfZ-(F)	CCATCATTCACGCCTGGGACTTCAT	7
orfW-(R)	TTCGTAATATGGCTGAGGATCATCTG	
int-(F)	GATAAAGAGATCAAAGCC	33
int-(R)	ATCTGCTTCCATGTGGG	
LJ-(F)	GTGAATCTTGATGAGACGCTCTG	33
LJ-(R)	GGTGAGCCAGGCTTATTTGGG	
RJ-(F)	TCGTTAGCGTGTTCGGTTCGCAGG	33
RJ-(R)	TGCTTTGTACCAGTCACAGATAG	
CTX		
cep-(F)	TCGTTAGCGTGTCGGTTCGCAGG	This study
cep-(R)	TGCTTTGTACCAGTCACAGATAG	
orfU-(F)	GCTACATGTTTAGCTCACTG	This study
orfU-(R)	AGGTGCGTTAGTCATCAGCG	
ace-(F)	GGCGTATTGTATCTATTAAA	This study
ace(R)	GGTGTTATTTGATGGCTGCATG	
zot-(R)	GGCGGTACGAGTAAAACAAATCC	This study
zot-(F)	GCTTATGATGGACACCCTTTA	
ctxA-(F)	TTTAACGCTCGCAGGGC	35
ctxA-(R)	GGGCGAGAAAGGACGC	
ctxB-(F)	GGTTGCTTCTCATCATCGAACCAC	7
ctxB-(R)	GATACACATAATAGAATTAAGGAT	
toxR		
toxR-(F)	ATGTTCGGATTAGGACAC	13
toxR-(R	TACTCACACACTTTGATGGC	

[a] F, forward primer; R, reverse primer.

right and left junction primers (Fig. 1) suggests that the VPI was likely to be integrated in the 10259 chromosome at sites identical to those of O1 strains (19, 22).

Sequence analysis data obtained so far suggest that the 10259 VPI differs from the VPIs of O1 strains primarily with respect to the *tcpA* gene, although minor variations could also be detected in the adjoining regions. In a recent study, Karaolis et al. (21) demonstrated that most of the divergence between the VPIs of 6th (classical) and 7th (El Tor) pandemic strains were located in or around the *tcpA* gene constituting the central region of VPI, but not its left or right segments. The authors hypothesized that this could arise as a result of a recombinational event involving genes located in the central segment. A similar mechanism might have contributed to generate the 10259 VPI, which shows an equal degree of divergence from both classical and El Tor biotype strains with respect to its *tcpA* and adjacent region sequences. If true, a crucial factor leading to such a recombinational event would be the generation of new variants of TcpA different from both classical or El Tor TcpA proteins. A comparative analysis (35) of four different TcpA variants (including the 10259 TcpA) revealed that the sequence variations were primarily in the carboxyl-terminal half of the 20-kDa protein with the majority of the variable or hypervariable residues located along the surface of TcpA, leading to alterations in their surface structures or epitopes (8). Such alterations are likely to influence the reactivity of the pilus or pilus protein to antibodies and/or ligands or receptors of biological importance.

The CTX genetic element of the strain 10259 was also probed with the primers for *ctxA*, *ctxB*, *zot*, *ace*, *orfU*, and *cep* genes, as listed in Table 2. All of these primers produced amplicons of the desired sizes, thereby documenting the presence of these genes in this non-O1 non-O139 strain. Amplicons generated with *ctxA* and *orfU* primers were subjected to sequencing analysis, and data were compared with those of O1 strains. The *ctxA* of 10259 showed only minimum changes from those of classical (0 bp) and El Tor (2 bp) strains. On the other hand, the 10259 *orfU* sequence diverged significantly from that of the classical strain by 56 bp (9.1% with 14 synonymous and 42 nonsynonymous changes), although it differed from the El

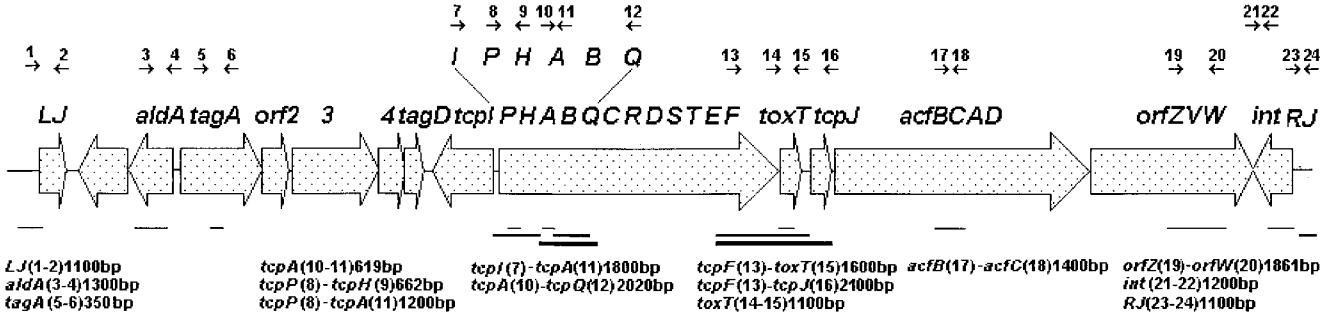

FIG. 1. Schematic representation of *V. cholerae* VPI with its right (RJ) and left (LJ) junctions. Amplicons generated with strain 10259 by using various primer pairs are shown with their respective sizes. Arrows represent forward and reverse primer pairs.

TABLE 3. Sequence divergence of VPI-associated genes of non-O1 non-O139 *V. cholerae* strain 10259 from those of O1 strains

Target gene(s) or intergenic region	Size (bp) of:		Divergence from O1 strains in bp (%)	
	Target sequence	Sequence compared	Classical[a]	E1 Tor[b]
aldA	1,300	1,180	8 (0.6)	9 (0.7)
tagA	350	315	1 (0.3)	2 (0.6)
tcpP-tcpH	662	610	6 (0.9)	7 (1.1)
Intergenic region (*tcpH-tcpA*)	598	302	10 (3.3)[c]	13 (4.3)
tcpA	619	590[d]	157 (26.0)[d]	169 (28.0)[d]
Intergenic region (*tcpF-toxT*)	207	187	2 (1.0)	2 (1.0)
toxT	1,100	588	7 (1.2)	17 (2.8)
acfB-acfC	1,400	963	5 (0.5)	9 (0.9)
int	1,200	955	3 (0.3)	9 (0.9)

[a] Based on accession no. X64098 and X74730.
[b] Based on accession no. AE 004168 and AE 004169.
[c] Reference 37.
[d] Reference 35.

Tor *orfU* by only 8 bp (1.3% with 6 synonymous and 2 nonsynonymous changes).

Recently, *orfU* has been proposed to be involved in the interaction of CTXϕ to its receptor TCP on the *V. cholerae* surface (5). A significant difference between the *orfU* sequences of classical and El Tor biotype strains was postulated to be responsible for the specific recognition of CTXϕs by biotype-specific TcpA proteins, the major structural unit of TCP. Therefore, the fact that the *orfU* sequence of 10259 shows close similarity to that of El Tor (but not of classical *orfU*), appears to be somewhat at variance to this concept, since 10259 TcpA was predicted to differ equally from both classical and El Tor TcpA proteins (Table 3). Our results are, however, in agreement with those obtained with certain other non-O1 non-O139 strains, the *orfU* sequences of which were shown to be more similar to those of El Tor than to those of classical strains (5). At least, two of these strains, 158 and 208, possessed a new variant of TcpA with significant differences from classical, El Tor, as well as 10259 TcpA (35). All of these considerations would suggest that the drift in the TcpA sequence in non-O1 non-O139 *V. cholerae* strains may not necessarily be related to the need to bind to different OrfU proteins for the acquisition of new type of CTXϕ. As a matter of fact, TcpA-independent acquisition of CTXϕ by *V. cholerae* under selective conditions has been documented recently (4, 13).

Documentation of the presence of VPI and CTX elements in strain 10259 and partial characterization of its virulence-associated genes have prompted us to address the question of their expression in relation to pathogenesis. Therefore, the expression of *toxR, tcpP/H*, *toxT*, *ctxA*, and *tcpA* genes was studied by reverse transcription-PCR (RT-PCR) with the set of primers listed in Table 1. Briefly, bacterial RNA was extracted with TRIZOL reagent (GIBCO-BRL) from cells grown under the appropriate culture conditions, and the extracted material was treated with RNase-free DNase (Ambion). Purified RNA was used to obtain cDNA. For this, 1 μg of RNA was mixed with 0.1 M dithiothreitol (DTT), 2 pmol of each primer of the primer pair, 10 mM deoxynucleotide triphosphate (dNTP), and 5× first-strand buffer in 9.5 μl of reaction volume, and the mixture was incubated at 42°C for 2 min followed by immediate cooling. Next, 100 U of the enzyme reverse transcriptase (GIBCO-BRL) was added to this mixture, which was incubated at 42°C for 50 min. The reaction was terminated by incubating the mixture at 70°C for 15 min. The cDNA preparation thus obtained was amplified by PCR by the methodology described earlier.

The results obtained in the RT-PCR experiments demonstrate that strain 10259, when grown in LB at pH 6.5 for 16 h at 30°C under mild shaking conditions, expressed transcripts for *toxR, tcpP/H*, *toxT*, and *tcpA*, but not for *ctxA* (Fig. 2A). Similar results were obtained when the organism was grown in the same LB medium under static conditions or in colonization factor antigen (CFA) agar plates (data not shown). In all of these experiments, the *V. cholerae* O1 classical strain (O395) was used as the positive control to document the expression of virulence gene-associated transcripts as mentioned above (Fig. 2A). The expression of these genes was also tested by growing strain 10259 in modified AKI medium (52), which is known to favor the expression of *ctxAB* in El Tor biotype strains. While the control El Tor strain AD48 expressed *toxR, tcpP/H*, *toxT*, *ctxA*, and *tcpA* when grown in AKI medium at 37°C at pH 7.8 for 4 h under static conditions followed by 2 h of shaking conditions (24), strain 10259 showed expression of *toxR* with only weakly detectable transcripts of *toxT* and *ctxA*, but none for *tcpP/H* or *tcpA* (Fig. 2B). Culture of strain 10259 for a longer period (4 h of static conditions plus 6 h of shaking conditions) also failed to produce elevated levels of transcripts for these genes (data not shown).

Failure to detect *ctxA* expression of 10259 in LB medium (which activated the transcription of both *toxR* and *toxT* genes) prompted us to examine the promoter proximal region located upstream of *ctxAB*, which contains the binding sites for ToxR and/or ToxT proteins (11, 27, 28, 39). Therefore, the region between the *zot* and *ctxA* genes in strain 10259 was amplified with the forward primer of the *zot* gene and a reverse one from the *ctxA* gene (Table 2). The amplified product of 1.4 kb, which contained the *zot-ctxA* intergenic region (124 bp) and parts of the *ctxA* and *zot* genes (434 and 923 bp, respectively), was sequenced, and the data were compared with the corresponding region sequence of *V. cholerae* O1 strain O395. The comparison revealed that while strain O395 contained seven copies of a heptanucleotide (TTTTGAT) repeat, strain 10259 contained only two such copies (Fig. 3). This difference appeared to be of considerable interest, since earlier studies (26) already demonstrated that the repeated sequence is required for the activation of the *ctxAB* promoter and a higher number of such tandem repeats produces higher levels of toxin. Apart from this, the *zot-ctxA* intergenic region sequence of 10259 did not show any other difference from that of the strain O395. However, two nucleotide changes were noted between these two strains in the partial (443 bp) *zot* sequences.

Based on the results presented so far, it is tempting to speculate that the observed lack of *ctxA* expression by strain 10259 under *toxR*- and *toxT*-expressing conditions could be attributed to the presence of only two copies of the heptanucleotide repeats, instead of the three to eight such copies that are reported to be present in the toxigenic O1 strains tested so far (26). To test this hypothesis, a recombinant of strain 10259 was

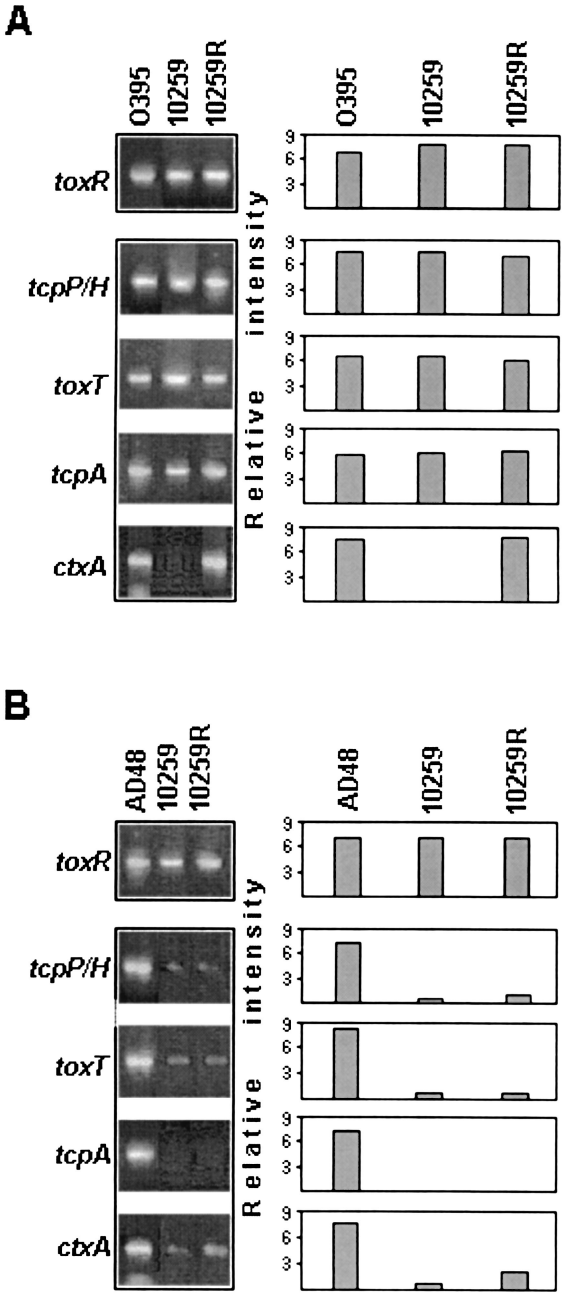

FIG. 2. Detection of *toxR*, *tcpP/H*, *toxT*, *tcpA*, and *ctxA* transcripts by RT-PCR in *V. cholerae* strains O395, AD48, 10259, and 10259R. Organisms were grown in LB medium at pH 6.5 at 30°C for 16 h under mild shaking conditions (A) and AKI medium at pH 7.8 at 37°C for 4 h under static conditions followed by 2 h of shaking conditions (B). Columns in the right-hand panel show the relative intensity of the bands (left-hand panel) determined by the densitometric analysis with the Molecular Analyst (version 1.5) software package.

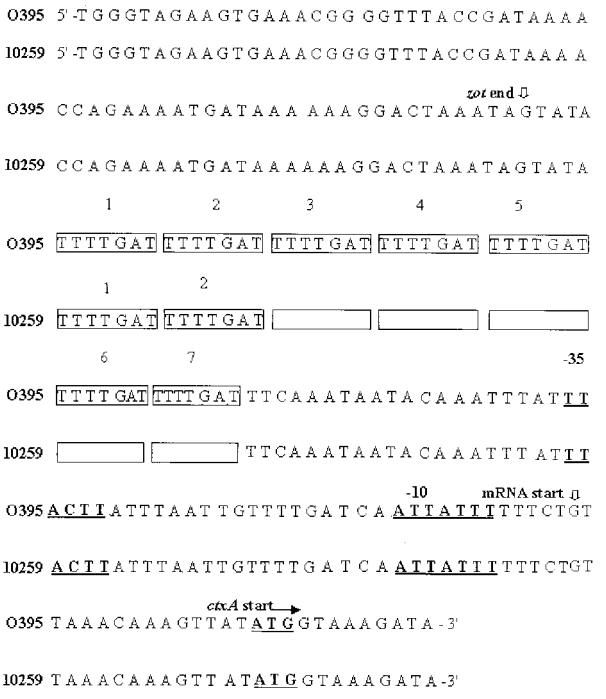

FIG. 3. Partial nucleotide sequence comparison of the *ctxAB* upstream regions of *V. cholerae* strains O395 and 10259. The sequences are aligned to show differences in the numbers of heptanucleotide repeats (in boxes).

generated through the replacement of its *zot-ctxA* intergenic region with the homologous region derived from *V. cholerae* O1 strain O395, which has seven copies of the TTTTGAT repeats. For this, the chromosomal DNA of O395 was amplified with the *zot* forward and *ctxA* reverse primers, and the resultant amplified product of 1.4 kb was directly cloned into a suicide vector, pCVD442 (12). Briefly, the plasmid pCVD442 was digested with *Sma*I, and a single 3′-T overhang (44) of the linearized product was generated by incubating 2 μg of the purified digested material with 10 mM dTTP, 50 mM $MgCl_2$, 5 U of *Taq* DNA polymerase, and 10× reaction buffer in a final reaction volume of 50 μl. An aliquot (20 μl) of the mixture was purified and mixed with 1.5 μg of the amplified product, and the mixture was then incubated at 23°C for 16 h in the presence of T4 DNA ligase (GIBCO-BRL). The ligated product (pCAS442) was used to transform *Escherichia coli* SM10λpir (29), and ampicillin-resistant transformants were selected. Transformed *E. coli* cells harboring the recombinant vector pCAS442 were allowed to conjugate with *V. cholerae* strain 10259 at a donor/recipient ratio of 1:10. Transconjugants of *V. cholerae* (harboring the chromosomally integrated vector) were selected on thiosulfate-citrate-bile-sucrose (TCBS) agar plates containing ampicillin (50 μg/ml) and checked for their reactivity to the antisera to the O53 serogroup. Next, selected transconjugant colonies were grown on Luria agar (LA) plates containing 10% (wt/vol) sucrose to select organisms to undergo a second recombinational event resulting in the deletion of the suicide vector sequence from the host chromosome. The recombinant colonies thus obtained (10259R) were further checked by their growth requirements, reactivity to O53-specific antisera, and sensitivity to ampicillin. Finally, the chromosomal DNA of 10259R was amplified with the *zot-ctxA* primer pair, and the product was sequenced to ensure that it contained seven repeats of the heptanucleotide sequence (TTTTGAT) at the proper position.

Following characterization, the recombinant strain 10259R

TABLE 4. CT production by *V. cholerae* strains grown under two different cultural conditions

Strain	Amt of CT[a] produced in medium (ng/ml/unit of opacity)	
	LB[b]	AKI[c]
O395	2,700	ND[d]
10259	2.8	16
10259R	2,650	53
AD48	ND	663
Co900	ND	1,035
V2	216	24
S7	1,800	80

[a] Assayed by the GM1 ELISA method (17) and expressed as nanograms of toxin per milliliter of culture supernatant per unit of opacity of bacterial suspension measured at 540 nm.
[b] LB medium, pH 6.5, incubation temperature of 30°C, 16 h of culture.
[c] AKI medium, pH 7.8, incubation temperature of 37°C, 4 h of static culture with limited aeration followed by 16 h of mild shaking culture with adequate aeration.
[d] ND, not determined.

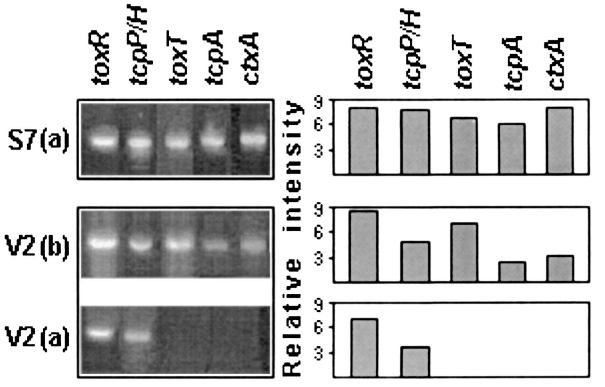

FIG. 5. Detection of *toxR*, *tcpP/H*, *toxT*, *tcpA*, and *ctxA* transcripts by RT-PCR in *V. cholerae* strains V2 and S7. Organisms were grown in LB medium at pH 6.5 at 30°C for 16 h (a) or 6 h (b) under mild shaking conditions. Columns in the right-hand panel show the relative intensity of the bands (left-hand panel) determined by densitometric analysis.

was tested for expression of virulence genes by RT-PCR as described earlier. When grown in LB at pH 6.5 at 30°C, the organism was shown to express *ctxA* in addition to *tcpP/H*, *toxR*, *toxT*, and *tcpA* (Fig. 2A). However, transcription of *tcpP/H*, *toxT*, and *ctxA* was detectable at low levels only in AKI medium-grown cells of strain 10259R (Fig. 2B). The RT-PCR data were extended through determination of the translated products CT and TcpA. Production of CT was estimated by GM1 enzyme-linked immunosorbent assay (ELISA) (17) with culture supernatants of cells grown under the appropriate culture conditions. While the wild-type strain 10259 produced a barely detectable level of CT in the LB-grown culture supernatant, the recombinant strain 10259R produced a significantly (about 1,000-fold) larger amount of CT in cultures grown under the same conditions (Table 4). In fact, CT produced by 10259R was quite comparable to that produced by the classical strain O395 in LB medium. Production of TcpA was also tested by immunoblotting experiments with a rabbit polyclonal antiserum to the protein. Both the wild-type and recombinant strains expressed TcpA when grown in LB medium, and the level of expression was quite comparable to that of the classical strain O395 grown under the same culture conditions (Fig. 4).

Expression of virulence genes in two other non-O1 non-O139 strains (V2 and S7) was also studied by RT-PCR. The strains, which were obtained from different sources, belonged to the same serogroup, O37 (Table 1), and possessed similar restriction fragment length polymorphism pattern on pulsed-field gel electrophoresis. Both of the strains contained VPI (with classical type of *tcpA*) and CTX-associated genes that were demonstrable by the PCR and/or DNA probe assay (14, 19; A. Sarkar and A. C. Ghose, unpublished data). When grown in LB medium, the strain S7 produced *tcpP/H*, *toxR*, *toxT*, *tcpA*, and *ctxA* transcripts (Fig. 5), which could be corroborated through the determination of its ability to produce CT and TcpA by ELISA (Table 4) and immunoblotting experiments (Fig. 4), respectively. On the other hand, only *tcpP/H* and *toxR* transcripts were detectable with the strain V2 grown for 16 h in LB (Fig. 5). Interestingly enough, transcripts of *toxT*, *ctxA*, and *tcpA* (in addition to those of *tcpP/H* and *toxR*) could be detected in V2 when grown for 6 h under the same culture conditions. These results are in apparent agreement with the low-level production of CT (Table 4) and TcpA (Fig. 4) by the strain as demonstrable by serological assays.

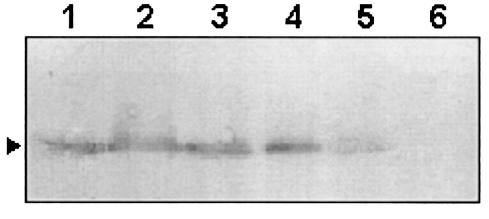

FIG. 4. Expression of TcpA by *V. cholerae* strains grown in LB medium (pH 6.5 at 30°C for 16 h) as determined by immunoblotting experiments with anti-TcpA serum. Whole-cell lysates of O395 (lane 1), 10259 (lane 2), 10259R (lane 3), S7 (lane 4), V2 (lane 5), and a Δ*tcpA* strain, V1228 (lane 6), were used as antigens. An arrowhead indicates 20-kDa TcpA bands.

Non-O1 non-O139 *Vibrio cholerae* strains harboring CT genes have been shown to produce considerably smaller amounts of CT than those produced by their O1 counterparts (10, 14, 32, 33). Several possible explanations may be provided for this, including (i) the absence of ToxT protein (due to the absence of VPI or *toxT* gene), which is known to be responsible for enhanced production of CT in *V. cholerae*; (ii) the presence of a *toxT* allele functionally deficient from the canonical *toxT*; (iii) poor production or a lack of production of ToxT as a result of defects or deviations in the signaling pathway that couple appropriate environmental signals to *toxT* activation; and (iv) failure to activate *ctxAB* operon (despite the production of an adequate amount of functional ToxT) due to the lack of a sufficient number of the heptanucleotide repeats in its promoter region.

Expression of *ctxA* in absence of any detectable transcripts of *tcpA* by strain 10259R in AKI medium raises an interesting question regarding the relative efficiency with which these two genes can be transcriptionally activated in *V. cholerae*. In a recent study, Yu and DiRita (55) have demonstrated that, although ToxT is the direct activator of both *ctxA* and *tcpA*, *ctxA* transcription regulation is much more complex than that

of *tcpA*. It is also suggested that the *ctxA* is under the control of a more efficient ToxT-dependent promoter compared to that of *tcpA*. Thus, under ToxT-limiting conditions, as is the case for strain 10259R grown in AKI medium, expression of *ctxA* may be achieved even in the absence of a detectable level of *tcpA* transcripts.

Coordinately regulated expression of CT and TcpA is an essential (although perhaps not sufficient) feature of epidemic-causing strains of *V. cholerae*, which so far has differentiated them from strains that are not associated with epidemics. Evidently, the genetic background of these strains plays a crucial role in this effect. The emergence of the O139 strain with epidemic potential from an O1 El Tor strain demonstrates that this feature may be retained by a non-O1 strain as well, provided it has a genetic makeup otherwise similar to that of O1 strains (50). Coordinate expression of CT and TcpA in the non-O1 non-O139 *V. cholerae* strain S7 described here also supports this concept, since this strain was likely to be derived from an O1 classical strain (3) and known to be involved in an epidemic in Sudan in 1968. Results obtained with the genetically engineered strain 10259R, however, demonstrate that coordinate expression of high levels of CT and TcpA is also achievable with a non-O1 non-O139 strain that may not be directly related to an epidemic strain as its origin. Although strain 10259R has shown its pathogenic potential in in vitro experiments, preliminary data generated in our laboratory have demonstrated considerable enhancement of its ability to produce toxin in vivo.

This work was supported by a grant [37(1008)/99-EMR-II] from the Council of Scientific and Industrial Research, Government of India.

The helpful technical assistance of Prabal Gupta and Debashis Mazumder is acknowledged.

REFERENCES

1. **Bagchi, K., P. Echeverria, J. D. Arthur, O. Sethabutr, O. Serichantalergs, and C. W. Hoge.** 1993. Epidemic diarrhea caused by *Vibrio cholerae* non-O1 that produced heat-stable toxin among Khmers in a camp in Thailand. J. Clin. Microbiol. **31:**1315–1317.
2. **Behari, J., L. Stagon, and S. B. Calderwood.** 2001. *pepA*, a gene mediating pH regulation of virulence genes in *Vibrio cholerae*. J. Bacteriol. **183:**178–188.
3. **Bik, E. M., R. D. Gouw, and F. R. Mooi.** 1996. DNA fingerprinting of *Vibrio cholerae* strains with a novel insertion sequence element: a tool to identify epidemic strains. J. Clin. Microbiol. **34:**1453–1461.
4. **Boyd, E. F., and M. K. Waldor.** 1999. Alternative mechanism of cholera toxin acquisition by *Vibrio cholerae*: generalized transduction of CTXΦ by bacteriophage CP-T1. Infect. Immun. **67:**5898–5905.
5. **Boyd, E. F., A. J. Heilpern, and M. K. Waldor.** 2000. Molecular analysis of a putative CTXϕ precursor and evidence for independent acquisition of distinct CTXϕs by toxigenic *Vibrio cholerae*. J. Bacteriol. **182:**5530–5538.
6. **Carroll, P. A., K. T. Tashima, M. B. Rogers, V. J. DiRita, and S. B. Calderwood.** 1997. Phase variation in *tcpH* modulates expression of the ToxR regulon in *Vibrio cholerae.* Mol. Microbiol. **25:**1099–1111.
7. **Chakraborty, S., A. K. Mukhopadhyay, R. K. Bhadra, A. N. Ghosh, R. Mitra, T. Shimada, S. Yamasaki, S. M. Faruque, Y. Takeda, R. R. Colwell, and G. B. Nair.** 2000. Virulence genes in environmental strains of *Vibrio cholerae*. Appl. Environ. Microbiol. **66:**4022–4028.
8. **Chattopadhyaya, R., and A. C. Ghose.** 2002. Model of *Vibrio cholerae* toxin coregulated pilin capable of filament formation. Protein Eng. **15:**297–304.
9. **Dalsgaard, A., M. J. Albert, D. N. Taylor, T. Shimada, R. Meza, O. Serichantalergs, and P. Echeverria.** 1995. Characterization of *Vibrio cholerae* non-O1 serogroups obtained from an outbreak of diarrhea in Lima, Peru. J. Clin. Microbiol. **33:**2715–2722.
10. **Datta-Roy, K., K. Banerjee, S. P. De, and A. C. Ghose.** 1986. Comparative study of expression of hemagglutinins, hemolysins, and enterotoxins by clinical and environmental isolates of non-O1 *Vibrio cholerae* in relation to their enteropathogenicity. Appl. Environ. Microbiol. **52:**875–879.
11. **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.
12. **Donnenberg, M. S., and J. B. Kaper.** 1991. Construction of an *eae* deletion mutant of enteropathogenic *Escherichia coli* by using a positive-selection suicide vector. Infect. Immun. **59:**4310–4317.
13. **Faruque, S. M., Asadulghani, A. R. M. A. Alim, M. J. Albert, K. M. N. Islam, and J. J. Mekalanos.**1998. Induction of the lysogenic phage encoding cholera toxin in naturally occurring strains of toxigenic *Vibrio cholerae* O1 and O139. Infect. Immun. **66:**3752–3757.
14. **Ghosh, C., R. K. Nandy, S. K. Dasgupta, G. B. Nair, R. H. Hall, and A. C. Ghose.** 1997. A search for cholera toxin (CT), toxin coregulated pilus (TCP), the regulatory element ToxR and other virulence factors in non-O1/non-O139 *Vibrio cholerae*. Microb. Pathog. **22:**199–208.
15. **Hase, C. C., and J. J. Mekalanos.** 1998. TcpP protein is a positive regulator of virulence gene expression in *Vibrio cholerae.* Proc. Natl. Acad. Sci. USA **95:**730–734.
16. **Heidelberg, J. F., J. A. Eisen, W. C. Nelson, R. A. Clayton, M. L. Gwinn, R. J. Dodson, D. H. Haft, E. K. Hickey, J. D. Peterson, L. Umayam, S. R. Gill, K. E. Nelson, T. D. Read, H. Tettelin, D. Richardson, M. D. Ermolavea, J. Vamathevan, S. Bass, H. Qin, I. Daragoi, P. Sellers, L. McDonald, T. Utterback, R. D. Fleishmann, W. C. Nierman, O. White, S. L. Salzberg, H. O. Smith, R. R. Colwell, J. J. Mekalanos, J. C. Venter, and C. M. Fraser.** 2000. DNA sequence of both chromosome of the cholera pathogen *Vibrio cholerae*. Nature **406:**477–484.
17. **Holmgren, J.** 1973. Comparison of the tissue receptors for *Vibrio cholerae* and *Escherichia coli* enterotoxins by means of gangliosides and natural cholera toxoid. Infect. Immun. **8:**851–859.
18. **Kaper, J. B., J. G. Morris, Jr., and M. M. Levine.** 1995. Cholera. Clin. Microbiol. Rev. **8:**48–86.
19. **Karaolis, D. K. R., J. A. Johnson, C. C. Bailey, E. C. Boedeker, J. B. Kaper, and P. R. Reeves.** 1998. A *Vibrio cholerae* pathogenicity island associated with epidemic and pandemic strains. Proc. Natl. Acad. Sci. USA **95:**3134–3139.
20. **Karaolis, D. K. R., S. Somara, D. R. Manaeval, Jr., J. A. Johnson, and J. B. Kaper.** 1999. A bacteriophage encoding a pathogenicity island, a type-IV pilus and a phage receptor in cholera bacteria. Nature **399:**375–379.
21. **Karaolis, D. K. R., R. Lan, J. B. Kaper, and P. R. Reeves.** 2001. Comparison of *Vibrio cholerae* pathogenicity islands in sixth and seventh pandemic strains. Infect. Immun. **69:**1947–1952.
22. **Kovach, M. E., M. D. Shaffer, and K. M. Peterson.** 1996. A putative integrase gene defines the distal end of a large cluster of ToxR-regulated colonization genes in *Vibrio cholerae*. Microbiology **142:**2165–2174.
23. **Krukonis, S. E., R. R. Yu, and V. J. DiRita.** 2000. The *Vibrio cholerae* ToxR/TcpP/ToxT virulence cascade: distinct roles for two membrane-localized transcriptional activators on a single promoter. Mol. Microbiol. **38:**67–84.
24. **Medrano, A. I., V. J. DiRita, G. Castillo, and J. Sanchez.** 1999. Transient transcriptional activation of the *Vibrio cholerae* El Tor virulence regulator ToxT in response to culture conditions. Infect. Immun. **67:**2178–2183.
25. **Mekalanos, J. J.** 1983. Duplication and amplification of toxin genes in *Vibrio cholerae*. Cell **35:**253–263.
26. **Mekalanos, J. J., D. J. Swartz, G. D. N. Pearson, N. Harford, F. Groyne, and M. de Wilde.** 1983. Cholera toxin genes: nucleotide sequence, deletion analysis and vaccine development. Nature **306:**551–557.
27. **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.
28. **Miller, V. L., R. K. Taylor, and J. J. Mekalanos.** 1987. Cholera toxin transcriptional activator ToxR is a transmembrane DNA binding protein. Cell **48:**271–279.
29. **Miller, V. L., and J. J. Mekalanos.** 1988. A novel suicide vector and its use in construction of insertion mutations: osmoregulation of outer membrane proteins and virulence determinants in *Vibrio cholerae* requires *toxR*. J. Bacteriol. **170:**2575–2583.
30. **Morris, J. G., Jr.** 1990. Non-O group 1 *Vibrio cholerae*: a look at the epidemiology of an occasional pathogen. Epidemiol. Rev. **12:**179–191.
31. **Morris, J. G., Jr.** 1994. Non-O group 1 *Vibrio cholerae* strains not associated with epidemic disease, p. 103–115. *In* I. K. Wachsmuth, P. A. Blake, and Ø. Olsvik (ed.), *Vibrio cholerae* and cholera: molecular to global perspectives. ASM Press, Washington, D.C.
32. **Mukhopadhyay, A. K., P. K. Saha, S. Garg, S. K. Bhattacharya, T. Shimada, T. Takeda, Y. Takeda, and G. B. Nair.** 1995. Distribution and virulence of *Vibrio cholerae* belonging to serogroups other than O1 and O139: a nation survey. Epidemiol. Infect. **114:**65–70.
33. **Mukhopadhyay, A. K., S. Chakraborty, Y. Takeda, G. B. Nair, and D. E. Berg.** 2001. Characterization of VPI pathogenicity island and CTXϕ prophage in environmental strains of *Vibrio cholerae*. J. Bacteriol. **183:**4737–4746.
34. **Nandi, B., R. K. Nandy, S. Mukhopadhyay, G. B. Nair, T. Shimada, and A. C. Ghose.** 2000. Rapid method for species-specific identification of *Vibrio cholerae* using primers targeted to the gene of outer membrane protein OmpW. J. Clin. Microbiol. **38:**4145–4151.
35. **Nandi, B., R. K. Nandy, A. C. P. Vicente, and A. C. Ghose.** 2000. Molecular

characterization of a new variant of toxin-coregulated pilus protein (TcpA) in a toxigenic non-O1/non-O139 strain of *Vibrio cholerae*. Infect. Immun. **68:**948–952.
36. **Novais, R. C., A. Coelho, C. A. Salles, and A. C. Vicente.** 1999. Toxin-co-regulated pilus cluster in non-O1, non toxigenic *Vibrio cholerae*: evidence of a third allele of pilin gene. FEMS Microbiol. Lett. **171:**49–55.
37. **Ogierman, M. A., E. Voss, C. Meaney, R. Faast, R. Attridge, and P. A. Manning.** 1996. Comparison of the promoter proximal regions of the toxin coregulated *tcp* gene cluster in classical and El Tor strains of *Vibrio cholerae* O1. Gene **170:**9–16.
38. **Pearson, G. D. N., A. Woods, S. L. Chiang, and J. J. Mekalanos.** 1993. CTX genetic element encodes a site specific recombination system and an intestinal colonization factor. Proc. Natl. Acad. Sci. USA **90:**3750–3754.
39. **Pfau, J. D., and R. K. Taylor.** 1996. Genetic footprint of the ToxR-binding site in the promoter for cholera toxin. Mol. Microbiol. **20:**213–222.
40. **Rivera, I. N. G., J. Chun, A. Huq, R. B. Sack, and R. R. Colwell.** 2001. Genotypes associated with virulence in environmental isolates of *Vibrio cholerae*. Appl. Environ. Microbiol. **67:**2421–2429.
41. **Rudra, S., R. Mahajan, M. Mathur, K. Kathuria, and V. Talwar.** 1996. Cluster of cases of clinical cholera due to *Vibrio cholerae* O10 in east Delhi. Indian J. Med. Res. **103:**71–73.
42. **Said, B., H. R. Smith, S. M. Scotland, and B. Rowe.** 1995. Detection and differentiation of the gene for toxin co-regulated pili (*tcpA*) in *Vibrio cholerae* non-O1 using the polymerase chain reaction. FEMS Microbiol. Lett. **125:** 205–210.
43. **Sharma, C., M. Thungapathra, A. Ghosh, A. K. Mukhopadhyay, A. Basu, R. Mitra, I. Basu, S. K. Bhattacharya, T. Shimada, T. Ramamurthy, T. Takeda, S. Yamasaki, Y. Takeda, and G. B. Nair.** 1998. Molecular analysis of non-O1, non-O139 *Vibrio cholerae* associated with an unusual upsurge in the incidence of cholera like disease in Calcutta, India. J. Clin. Microbiol. **36:**756–763.
44. **Shuman, S.** 1994. Novel approach to molecular cloning and polynucleotide synthesis. J. Biol. Chem. **269:**32678–32684.
45. **Skorupski, K., and R. K. Taylor.** 1997. Control of the ToxR virulence regulon in *Vibrio cholerae* by environmental stimuli. Mol. Microbiol. **25:** 1003–1009.
46. **Skorupski, K., and R. K. Taylor.** 1999. A new level in the *Vibrio cholerae* ToxR virulence cascade: AphA is required for transcriptional activation of the *tcpPH* operon. Mol. Microbiol. **31:**763–771.
47. **Taylor, R., C. Shaw, K. Peterson, P. Spears, and J. Mekalanos.** 1988. Safe, live *Vibrio cholerae* vaccines? Vaccine **6:**151–154.
48. **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.
49. **Voss, E., P. A. Manning, and S. T. Attridge.** 1996. The toxin-coregulated pilus is a colonization factor and protective antigen of *Vibrio cholerae* El Tor. Microb. Pathog. **20:**141–153.
50. **Waldor, M. K., and J. J. Mekalanos.** 1994. ToxR regulates virulence gene expression in non-O1 strains of *Vibrio cholerae* that cause epidemic cholera. Infect. Immun. **62:**72–78.
51. **Waldor, M. K., and J. J. Mekalanos.** 1996. Lysogenic conversion by a filamentous phage encoding cholera toxin. Science **272:**1910–1914.
52. **Waldor, M. K., E. J. Rubin, G. D. N. Pearson, H. Kimsey, and J. J. Mekalanos.** 1997. Regulation, replication, and integration functions of the *Vibrio cholerae* CTX are encoded by region RS2. Mol. Microbiol. **24:**917–926.
53. **Yamai, S., T. Okitsu, T. Shimada, and Y. Katsube.** 1997. Distribution of serogroups of *Vibrio cholerae* nonO1/nonO139 with specific reference to their ability to produce cholera toxin and addition of novel serogroups. Jpn. J. Assoc. Infect. Dis. **71:**1037–1045.
54. **Yu, R. R., and V. J. DiRita.** 1999. Analysis of an autoregulatory loop controlling ToxT, cholera toxin, and toxin-coregulated pilus production in *Vibrio cholerae.* J. Bacteriol. **181:**2584–2592.
55. **Yu, R. R., and V. J. DiRita.** 2002. Regulation of gene expression in *Vibrio cholerae* by ToxT involves both the antirepression and RNA polymerase stimulation. Mol. Microbiol. **43:**119–134.

Editor: V. J. DiRita