INFECTION AND IMMUNITY, Apr. 1997, p. 1561–1565
0019-9567/97/$04.00+0

Vol. 65, No. 4

Promoter Activities in *Vibrio cholerae* ctxΦ Prophage

RAFAEL FANDO,[1] JOSÉ L. PÉREZ,[2] BORIS L. RODRIGUEZ,[1] JAVIER CAMPOS,[1] ALMA ROBERT,[1] LUIS GARCÍA,[2] ANISIA SILVA,[3] AND JORGE A. BENITEZ[1]*

Centro Nacional de Investigaciones Científicas,[1] Instituto Finlay, Sueros y Vacunas,[2] and Departamento de Microbiología, Facultad de Biología de la Universidad de La Habana,[3] Havana, Cuba

Received 31 May 1996/Returned for modification 7 August 1996/Accepted 17 January 1997

Comparison of cholera toxin (CT) production directed by different gene constructs and S1 nuclease mapping revealed the presence of a *ctxB*-specific promoter within the *ctxA* coding sequence. Initiation of transcription in this region occurred in wild-type El Tor and classical biotype choleragenic vibrios. We propose that transcription from the *ctxB*-specific promoter and a stronger ribosomal binding site on the *ctxB* mRNA synergistically contribute to achieve the correct (5B:1A) subunit stoichiometry. Plasmid pB, a CT promoterless vector expressing only CTB, was used to detect promoter activity by restoration of A-subunit synthesis. Promoter activity expressed in vitro and in vivo was detected upstream of the zonula occludens toxin gene, suggesting that this factor could be produced in vivo to contribute to fluid accumulation. No promoter activity was detected in vitro and in vivo upstream from the accessory cholera enterotoxin gene.

Vibrio cholerae strains of serogroups O1 and O139, the causative agents of cholera, secrete a potent enterotoxin, cholera toxin (CT), that causes the severe diarrhea of cholera (7). CT is an oligomeric protein composed of one A subunit (CTA) (28 kDa) that catalyzes NAD-dependent ADP-ribosylation and five B subunits (11.5 kDa) that bind to ganglioside GM_1, the holotoxin receptor, in the gut (7). The *ctxA* and *ctxB* genes are positively controlled by the product of *toxR*, a 38-kDa transmembrane DNA-binding protein (15, 16, 18).

Despite the abundant literature on the structure of CT, there is no satisfactory explanation for how the ratio of five CTB molecules to one CTA molecule is determined. Although early studies suggested that a stronger ribosomal binding site on the *ctxB* mRNA might be involved (13, 20, 21), it is not clear if this difference alone is responsible for subunit stoichiometry.

V. cholerae can produce other potentially toxic factors like the zonula occludens toxin (ZOT) (1, 6) and the accessory cholera enterotoxin (ACE) (27) that have been defined by their effects on rabbit ileal tissue mounted in Ussing chambers (6, 27). The genes encoding a core-encoded pilus (*cep*) (22), an open reading frame (*orfU*), ACE (*ace*), ZOT (*zot*), CTA (*ctxA*), and CTB (*ctxB*) are located in a transposon-like structure initially called the virulence cassette (14, 27). The virulence cassette is the prophage state of the ctxΦ filamentous phage (28). The products of *cep*, *ace*, *orfU*, and *zot* have been proposed to be involved in phage morphogenesis (28). These observations cast doubt on the potential of the *zot* and *ace* gene products to contribute to intestinal fluid accumulation.

In the present work, we detected the existence of a promoter activity upstream from the *ctxB* coding sequence (within the CTA open reading frame) and show that the *zot* regulatory region encodes a promoter activity that is expressed in vivo.

The structures of the plasmid constructs are shown in Fig. 1. These vectors were constructed by using standard recombinant DNA techniques and *Escherichia coli* XL1 as a host (24). Plasmid pBB6 contains a *Pst*I-*Eco*RI fragment from *V. cholerae* 569B encoding *ace*, *zot*, *ctxA*, and *ctxB* cloned in pBR322 (1). pCT contains a *Pst*I-*Hin*dIII fragment encoding *ace*, *zot*, *ctxA*, and *ctxB* subcloned from pBB6 into pUC18. Plasmid pA was constructed by first subcloning the 5′ *Pst*I-*Xba*I fragment of pCT in pUC19, deleting the region between the extreme *Bcl*I sites, blunting, insertion of a *Bam*HI octameric linker, and cloning back the 3′ *Xba*-*Hin*dIII region. pB was constructed in several steps. First, a *Bam*HI-*Hin*dIII fragment was transferred from pA to Bluescript SK (+) to create vector pIC, and a *Dra*I site was created by oligonucleotide-directed mutagenesis (Fig. 1A). Second, an *Eco*RI fragment containing the *rrnB* T_1T_2 transcription terminator (3) was cloned in pUC19 to produce pUCTTD (not shown). Third, pUCTTD was digested with *Hin*cII-*Hin*dIII, and the *Dra*I-*Hin*dIII fragment of pIC was cloned downstream from *rrnB* T_1T_2 to create pB. pC and pD were obtained by subcloning *Sca*I-*Hin*dIII and *Cla*I-*Hin*dIII fragments, respectively, from pB into pUCTTD digested with *Hin*cII and *Hin*dIII. The relevant constructs are displayed in Fig. 1A so that the deleted sequences can be appreciated as empty regions.

Each plasmid was introduced in *V. cholerae* 81 (2, 23). Strain 81 is a Δ(*cep orfU ace zot ctxA ctxB*) derivative of *V. cholerae* C7258 (El Tor, Ogawa, Peru, 1991) (2, 23). *V. cholerae* strains were grown on LB medium at 37°C, and ampicillin (100 μg/ml) was added when necessary. Alternatively, vibrios were grown in AKI cultures (12) and syncase medium (8). Production of CT was measured for each transformant by using monoclonal antibodies (MAbs) 4E1G5 (referred to as CT_B in ratios) and 1G10G5 (referred to as CT_A in ratios), directed against CTB and CTA from *V. cholerae* 569B, respectively. The concentration of toxin produced was determined in a GM_1 enzyme-linked immunosorbent assay (ELISA) (10), using a standard curve constructed with purified CT from *V. cholerae* 569B Inaba (a gift of Richard A. Finkelstein, University of Missouri—Columbia School of Medicine). A homogeneous solution of CT should yield the same amount of CT whether measured with one or the other MAb. A CT_B/CT_A ratio of >1 is interpreted to reflect the existence of an excess of B subunits that can assemble into pentameric CTB and be detected in the GM_1 ELISA only with MAb 4E1G5. It has been shown that CTB can assemble into a pentamer in the absence of CTA (25).

Since *ctxA* and *ctxB* constitute an operon under positive regulation by the products of *toxR*, *toxS*, and *toxT* (5), deletion of its 5′ regulatory region would be expected to have polar

* Corresponding author. Mailing address: Centro Nacional de Investigaciones Científicas, P.O. Box 6990, La Habana, Cuba. Fax: (537) 33 0497.

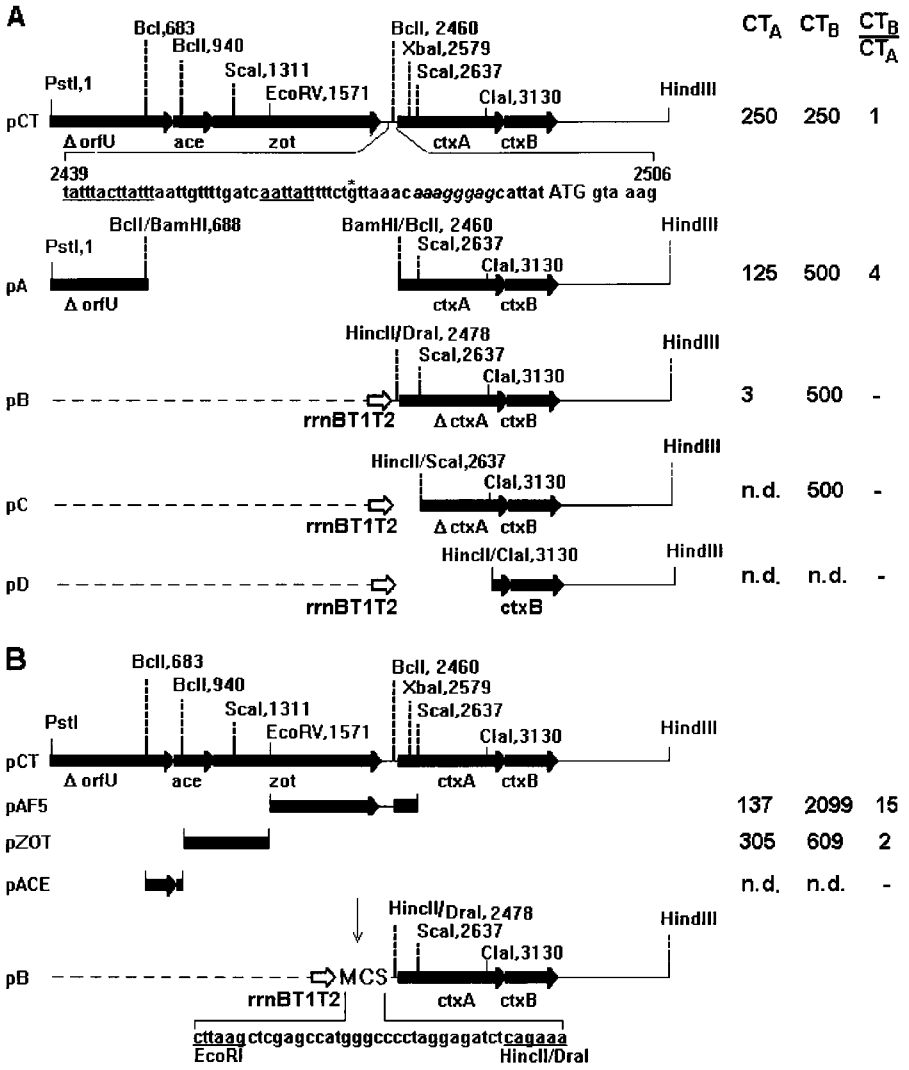

FIG. 1. (A) Deletion analysis of *V. cholerae* 569B ctxΦ prophage. Nucleotides are numbered starting at the *Pst*I site, and the plasmids are aligned so that empty spaces indicate deleted sequences. (B) Promoter activities in the *V. cholerae* ctxΦ prophage. The fragments indicated under pCT were cloned in the shown orientation in the multiple cloning site (MCS) preceding *ctxA* in pB to produce pAF5, pZOT, and pACE. Symbols and abbreviations: thin line, *V. cholerae* chromosomal DNA; interrupted line, pUC19 vector sequence; rrnBT1T2, transcription terminator; n.d., not detectable. The expanded region of the *toxR*-regulated promoter shows the −35 and −10 sequences underlined, the ribosomal binding site region in italics, and the *ctxA* start codon in uppercase. The G marked with an asterisk was mutated to T by oligonucleotide-directed mutagenesis to construct pB. CT_A and CT_B refer to the amounts of CT (nanograms per milliliter) measured with MAbs directed to the A and B subunits, respectively, by GM_1 ELISA. Each value is the mean of at least six independent LB cultures of strain 81 transformed with each plasmid. The standard error ranged from 5 to 17%.

effects on *ctxA* and *ctxB* expression. However, as shown in Fig. 1A, deletion of most of the regulatory region upstream from *ctxA* in pA diminished production of CTA and increased production of CTB, as judged from a CT_B/CT_A ratio of 4. Two other deletions removing the *ctxA* promoter −10 and −35 consensus sequences (pB) and CTA N-terminal sequences (pC) eliminated CTA production when placed downstream from the *rrnB*T_1T_2 transcription terminator without affecting CTB production (Fig. 1A).

In a second set of experiments, pB was used to detect putative promoters. Regulatory regions of the so-called virulence cassette were cloned into the multiple cloning site lying between the transcription terminator and the CTA-coding sequence. Insertion of the *Eco*RV-*Sca*I fragment containing the entire *ctxA* regulatory region in vector pAF5 restored CTA production but with a high CT_B/CT_A ratio (Fig. 1B). It should be noted that this manipulation created a 159-bp insertion immediately upstream of the *ctxA* coding sequence. Insertion of a *Bcl*I-*Eco*RV fragment encoding the C terminus of *ace* and the N terminus of *zot* in pZOT also restored CTA production, revealing the existence of promoter activity in this region (Fig. 1B). On the contrary, cloning of a *Bcl*I fragment containing the C terminus of *orfU* and the N terminus of *ace* in pACE did not restore CTA production, suggesting the absence of promoter activity in this region (Fig. 1B). ACE was discovered by subcloning fragments of the virulence cassette in an expression vector and assessing the capacity of each subclone to elicit changes in short-circuit current and/or resistance in Ussing chambers (27). Thus, it is likely that ACE could have been expressed in these studies from the *lac* promoter. Our results suggest that *ace* either is tightly repressed or is part of an operon under the control of an upstream promoter.

The following conclusions can be reached from the forego-

TABLE 1. CTB subunit production from the *ctxB*-specific promoter

Strain (n^a)	Mean CTB production (ng/ml) ± SD		
	Syncase	AKI	LB
C7258 (5)	ND[b]	180 ± 15	ND
72 (10)	250 ± 10	0.2	7 ± 1

[a] *n*, number of independent cultures analyzed.
[b] ND, not detectable.

ing experiments. First, the data from Fig. 1A indicate the existence of a *ctxB*-specific promoter in the CTA-coding sequence located within the *Sca*I and *Cla*I sites in *ctxA* (Fig. 1A, pC and pD). Second, if the *toxR*-regulated promoter is partly or entirely removed, the *ctxB*-specific promoter still allows CTB synthesis (Fig. 1A, pA, pB, and pC). Furthermore, insertion of DNA sequences that increase the distance between the *toxR*-regulated promoter and the *ctxB*-specific promoter enhanced the latter, leading to an excess of pentameric CTB (Fig. 1B, pAF5). Thus, proper positioning of the *toxR*-regulated promoter and *ctx* coding sequence is required to avoid overproduction of CTB subunits.

We have constructed a derivative of strain 81 containing the *rrnB*T_1T_2-Δ(*ctxA* ctxB) cartridge from pC integrated in its *hap* locus, encoding hemagglutinin-protease (9). First, a *Pvu*II fragment containing the *rrnB*T_1T_2-Δ(*ctxA ctxB*) construct was cloned in the unique *Stu*I site located in the *hap* gene carried in plasmid pCH2 (9). Next, the *hap*::*rrnB*T_1T_2-Δ(*ctxA ctxB*) allele was transferred to the suicide vector pGP704 (17) and mobilized from *E. coli* SM10λpir (26) to strain 81, and an ampicillin-resistant cointegrate was selected. The cointegrate was grown in LB medium, and ampicillin-sensitive segregants were selected. Strain 72, a segregant in which the *hap* gene was replaced by *hap*::*rrnB*T_1T_2-Δ(*ctxA ctxB*), was isolated and characterized by Southern blot hybridization (data not shown). Strain C7258 produced maximal amounts of CTB in AKI cultures, while production by strain 72 was favored in syncase medium (Table 1). CTB production in strain 72 confirmed the functionality of the *ctxB*-specific promoter that responded differently to culture medium.

We introduced plasmid pC in regulatory mutants to determine if this promoter is controlled by *toxR* or *toxT*. Table 2 shows that pC restored CTB production in mutant strains JJM43 (*toxR*) and VJ740 (*toxT*). We conclude that the *ctxB*-specific promoter is not under positive control by the products of *toxR* and *toxT*. The *ctxB*-specific promoter could function in the wild-type vibrio to produce a transcript that could be translated into CTB molecules. The detection of CT mRNA of a size smaller than expected for an intact polycistronic transcript of the CT operon (19) favors this hypothesis.

To test this hypothesis, we purified total RNA from strains

TABLE 2. Production of CT in regulatory mutants

Strain	Amt (ng/ml)[a]	
	CT_A	CT_B
O395 (wild type, classical, Ogawa)	2,000	1,997
JJM43 (ToxR$^-$)	ND[b]	2
VJ740 (ToxT$^-$)	4	7
JJM43/pC	ND	16,000
VJ740/pC	ND	1,980

[a] Each value is the average of three independent cultures in syncase medium.
[b] ND, not detectable.

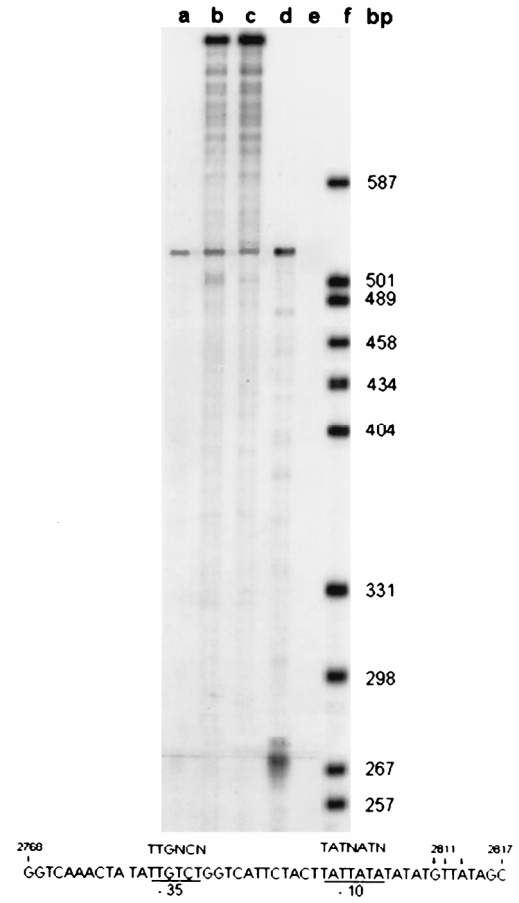

FIG. 2. S1 nuclease protection assay. Total RNA (50 μg) from each strain was hybridized to 10^5 cpm of radiolabeled probe and digested with 200 U of S1 nuclease, and protected fragments were sized in a 3% acrylamide sequencing gel (lane a, 72; lane b, 569B; lane c, C7258; lane d, 81 transformed with pB; lane e, untransformed 81). A *Hae*III-*Hpa*II digest of plasmid pUC19 was dephosphorylated, labeled with [γ-^{32}P]ATP, and used as a molecular weight marker (lane f). The DNA sequence of the *ctxB*-specific promoter region is shown below. Putative starts are indicated with arrows. The −10 and −35 regions are underlined, and the consensus sequence is displayed above them. Nucleotides are numbered starting at the *Pst*I site shown in Fig. 1.

72, 569B, C7258, 81 transformed with pB, and 81. A radiolabeled probe was synthesized by PCR using a 21-mer synthetic primer complementary to the *ctxB* sense strand. The 5′ end of this primer corresponds to the 69th nucleotide downstream from the CTB start codon (nucleotide 3240 in Fig. 1). One hundred picomoles of primer was extended to the conserved *Xba*I site of *ctxA* and labeled in 30 cycles (1 min at 94°C, 1 min at 55°C, and 1 min at 72°C) with *Taq* DNA polymerase and [α-^{32}P]dATP (50 μCi). The extended probe was purified by alkaline agarose gel electrophoresis (24). Each RNA was hybridized with radiolabeled probe and digested with S1 nuclease, and protected fragments were sized in a polyacrylamide sequencing gel (24).

A single protected fragment corresponding to a start at nucleotide 2811 ± 4 was detected in strain 72 (Fig. 2, lane a). In wild-type *V. cholerae*, the whole probe was protected due to transcription initiation at the *toxR*-regulated promoter (Fig. 2, lanes b and c). Nevertheless, initiation of transcription at 2811 also occurred in the wild-type vibrios (Fig. 2, lanes b and c). We observed two protected fragments in strain 81 transformed with plasmid pB (Fig. 2, lane d). The second fragment corre-

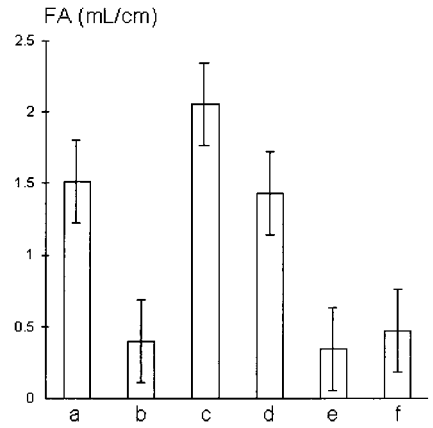

FIG. 3. Fluid accumulation (FA) per length of ligated rabbit ileal segment inoculated with strain 81 transformed with different plasmids. Columns: a, pCT; b, pB; c, pAF5; d, pZOT; e, pACE; f, untransformed 81. Each value represents the mean of at least five loops.

sponded to a start at nucleotide 3069. The significance of this second start, observed only when the *ctxB* gene was carried on a multicopy plasmid, is not clear. As expected, no protection was observed with total RNA from strain 81 (Fig. 2, lane e). In Fig. 2, we show the DNA sequence of the *ctxB*-specific promoter. This sequence is present in all *V. cholerae ctxA* genes sequenced (13, 20, 21). Since the start is over 90% of the time a purine, the actual RNA start in the *ctxB*-specific promoter is likely to be the G preceding nucleotide 2811 or the A 2 bp downstream. A −10 and −35 consensus sequence could be located upstream from the putative RNA start points (Fig. 2). Transcripts originating within the *ctxA* open reading frame in choleragenic vibrios can be translated only into CTB molecules and favor the fivefold excess of CTB over CTA subunits in the mature holotoxin. It should be noted that these transcripts carry the stronger *ctxB* ribosomal binding site (13, 20, 21). We propose that a stronger ribosomal binding site on the *ctxB* mRNA and transcription from the *ctxB*-specific promoter synergistically contribute to achieve the correct subunit stoichiometry.

Ileal loops (4, 11) were used to detect promoter activity in vivo. Approximately 10^8 CFU of strain 81 transformed with different plasmids was injected into ligated ileal segments of New Zealand adult rabbits, and fluid accumulation, due to restoration of CTA production, was measured. As shown in Fig. 3, the promoter activity located upstream from the *zot* structural gene in pZOT was active in vivo. No promoter activity could be detected in vivo upstream from *ace* coding sequence. ZOT has been shown to loosen the tight junctions between epithelial cells in rabbit ileal tissue mounted in Ussing chambers (1, 6). Our results suggest that ZOT could be expressed in vivo and contribute to the diarrheagenicity of infection with virulent vibrios. It should be noted that the *zot* promoter in pZOT could have escaped proper regulation due to an increase in copy number. In such a case, an increase in *zot* gene dosage in strains harboring the replication factor of the ctxΦ phage could suffice to endow vibrios with the ability to synthesize ZOT and secrete it to the extracellular medium.

We are grateful to Richard A. Finkelstein and Gregory A. McDonald for their comments and James B. Kaper and V. DiRita for providing plasmids and strains. We are indebted to José de La Fuente and Bianca García (Centro de Ingeniería Genética y Biotecnología, Havana) for their advice and assistance in S1 nuclease mapping. Reynaldo Oliva (Instituto Finlay, Havana) assisted in the ileal loop experiments.

REFERENCES

1. **Baudry, B., A. Fasano, J. M. Ketley, and J. B. Kaper.** 1992. Cloning of a gene (*zot*) encoding a new toxin produced by *Vibrio cholerae*. Infect. Immun. **60:**428–434.
2. **Benítez, J. A., A. J. Silva, B. L. Rodríguez, R. Fando, J. Campos, A. Robert, H. García, L. García, J. L. Pérez, R. Oliva, C. A. Torres, and T. Ledón.** 1996. Genetic manipulation of *Vibrio cholerae* for vaccine development: construction of live attenuated El Tor candidate vaccine strains. Arch. Med. Res. **27:**275–283.
3. **Brosius, J., T. J. Dull, D. D. Sleeter, and H. F. Noller.** 1981. Gene organization and primary structure of a ribosomal RNA operon from Escherichia coli. J. Mol. Biol. **148:**107–127.
4. **De, S. N., and D. N. Chatterje.** 1953. An experimental study of the mechanism of action of *Vibrio cholerae* on the intestinal mucous membrane. J. Pathol. Bacteriol. **66:**559–562.
5. **DiRita, V.** 1992. Co-ordinate expression of virulence genes by toxR in Vibrio cholerae. Mol. Microbiol. **6:**451–458.
6. **Fasano, A., B. Baudry, D. W. Pumplin, S. S. Wasserman, B. D. Tall, J. M. Ketley, and J. B. Kaper.** 1991. *Vibrio cholerae* produces a second enterotoxin which affects intestinal tight junctions. Proc. Natl. Acad. Sci. USA **88:**5242–5246.
7. **Finkelstein, R. A.** 1992. Cholera enterotoxin (choleragen): a historical perspective, p. 155–187. *In* D. Barua, and W. B. Greenough (ed.), Cholera. Plenum Medical Book Company, New York, N.Y.
8. **Finkelstein, R. A., P. Atthasampunna, M. Chulasamaya, and P. Charunmethee.** 1966. Pathogenesis of experimental cholera. Biologic activities of purified procholeragen A. J. Immunol. **96:**440–449.
9. **Häse, C. C., and R. A. Finkelstein.** 1991. Cloning and nucleotide sequence of the *Vibrio cholerae* hemagglutinin/protease (HA/protease) gene and construction of an HA/protease-negative strain. J. Bacteriol. **173:**3311–3317.
10. **Holmgren, J.** 1973. Comparison of the tissue receptors for *Vibrio cholerae* and *Escherichia coli* enterotoxins by means of gangliosides and natural cholera toxoids. Infect. Immun. **8:**851–859.
11. **Ichinose, Y., K. Yamamoto, N. Nakasone, M. J. Tanabe, T. Takeda, T. Miwanati, and M. Iwanaga.** 1987. Enterotoxicity of the El Tor-like hemolysin of non-O1 *Vibrio cholerae*. Infect. Immun. **55:**1090–1093.
12. **Iwanaga, M., K. Yamamoto, N. Higa, Y. Ichinose, N. Nakasone, and M. Tanabe.** 1986. Culture conditions for stimulating cholera toxin production by *Vibrio cholerae* O1 El Tor. Microbiol. Immunol. **30:**1075–1083.
13. **Lockman, H. A., J. E. Galen, and J. B. Kaper.** 1984. *Vibrio cholerae* enterotoxin genes: nucleotide sequence analysis of DNA encoding ADP-ribosyltransferase. J. Bacteriol. **159:**1086–1089.
14. **Mekalanos, J. J.** 1983. Duplication and amplification of toxin genes in *Vibrio cholerae*. Cell **35:**253–263.
15. **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.
16. **Miller, V. L., and J. J. Mekalanos.** 1985. Genetic analysis of the cholera toxin positive regulator ToxR. J. Bacteriol. **163:**580–585.
17. **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.
18. **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.
19. **Mishra, L., and R. K. Holmes.** 1987. Transcription of cholera toxin operon in wild-type and mutant strains of *Vibrio cholerae*. Infect. Immun. **55:**1529–1532.
20. **Pearson, G. D. N., and J. J. Mekalanos.** 1982. Molecular cloning of *Vibrio cholerae* enterotoxin genes in *Escherichia coli* K-12. Proc. Natl. Acad. Sci. USA **79:**2976–2980.
21. **Pearson, G. D. N., D. J. Swartz, J. J. Mekalanos, N. Harford, F. Groyne, and M. de Wilde.** 1983. Cholera toxin genes: nucleotide sequence, deletion analysis and vaccine development. Nature **306:**551–557.
22. **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.
23. **Robert, A., A. Silva, J. A. Benítez, B. L. Rodríguez, R. Fando, J. Campos, D. K. Sengupta, M. Boesman-Finkelstein, and R. A. Finkelstein.** 1996. Tagging a *Vibrio cholerae* El Tor candidate vaccine strain by disruption of its hemagglutinin/protease gene using a novel reporter enzyme: *Clostridium thermocellum* endoglucanase A. Vaccines **14:**1517–1522.
24. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.

25. **Simon, J., S. Hardy, J. Holmgren, S. Johansson, J. Sánchez, and T. R. Hirst.** 1988. Coordinated assembly of multisubunit proteins: oligomerization of bacterial enterotoxins in vivo and in vitro. Proc. Natl. Acad. Sci. USA **85:** 7109–7113.
26. **Simon, R., U. Priefer, and A. Puhler.** 1983. A broad host-range mobilization system for in vivo genetic engineering: transposon mutagenesis in Gram-negative bacteria. Bio/Technology **1:**784–791.
27. **Trucksis, M., J. E. Galen, J. Michalski, A. Fasano, and J. B. Kaper.** 1993. Accessory cholera enterotoxin (Ace). The third member of a *Vibrio cholerae* virulence cassette. Proc. Natl. Acad. Sci. USA **90:**5267–5271.
28. **Waldor, M. K., and J. J. Mekalanos.** 1996. Lysogenic conversion by a filamentous phage encoding cholera toxin. Science **272:**1910–1914.

Editor: P. E. Orndorff