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Construction and Characterization of Recombinant *Vibrio cholerae* Strains Producing Inactive Cholera Toxin Analogs

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The catalytic A subunit of cholera toxin (CT-A) is capable of ADP-ribosylating the guanine nucleotide-binding protein, which regulates cell adenylyl cyclase, leading to the life-threatening diarrhea of cholera. Amino acids involved in the enzymatic activity of CT-A have previously been identified. By means of site-directed mutagenesis, an analog of the CT-A subunit gene was created with codon substitutions for both Arg-7 and Glu-112, each of which has been shown to produce subunits lacking ADP-ribosyltransferase activity. The mutated gene fragment was exchanged for the wild-type copy in the previously cloned *ctxAB* operon from El Tor biotype, Ogawa serotype *Vibrio cholerae* strain 3083, which produces CT-2. Further, the zonula occludens toxin gene, *zot*, was inactivated by an insertional mutation to create the new plasmid construct pCT-2*. Additionally, a DNA fragment encoding the B subunit of CT-1 (CT produced by classical biotype, Inaba serotype *V. cholerae* strain 569B) was exchanged for the homologous part in pCT-2*, resulting in the creation of pCT-1*. These plasmid constructs were introduced into the CT-negative *V. cholerae* mutant strain JBK70 (El Tor biotype, Inaba serotype); CT-A^-B^+ derivatives CVD101 and CVD103 of classical biotype Ogawa and Inaba serotype strains 395 and 569B, respectively; El Tor biotype Inaba and Ogawa serotype strains C6706 and C7258, respectively, recently isolated in Peru; and O139 (synonym Bengal) strain SG25-1 from the current epidemic in India. Recombinant toxins (CT-1* and CT-2*), partially purified from culture supernatants of transformed JBK70, were shown to be inactive on mouse Y1 adrenal tumor cells and in an in vitro ADP-ribosyltransferase assay. CT-1* and CT-2* reacted with polyclonal and monoclonal antibodies against both A and B subunits of CT. The toxin analogs reacted with antibodies against CT-A and CT-B on cellulose acetate strips and in a G_{M1} enzyme-linked immunosorbent assay; they reacted appropriately with B-subunit epitype-specific monoclonal antibodies in checkerboard immunoblots, and they formed precipitin bands with G_{M1}-ganglioside in Ouchterlony tests. However, the reactions of the modified proteins with anti-A-subunit monoclonal antibodies were weaker than the reactions with wild-type holotoxins. *V. cholerae* strains carrying *ctxA, with either *ctxB-1* or *ctxB-2*, and inactivated *zot* genes were created by homologous recombination. The recombinant strains and the purified toxin analogs were inactive in the infant rabbit animal model. These strains may have use as attenuated live vaccines; the analog toxins themselves might have important applications in conjugate vaccines as well as in structure-function studies.**

Despite the availability of cholera vaccines in various forms since shortly after the first isolation of cholera vibrios by Robert Koch in 1883, a suitable cholera vaccine—one which is highly protective, economical, convenient, and without side effects—remains to be developed and deployed (reviewed in references 13, 14, and 27). The most promising current candidates include either living attenuated or killed vaccines administered perorally; both kinds have been evaluated in human clinical studies and found to be less than ideal in one or more aspects. A conjugate vaccine, consisting of detoxified vibrio lipopolysaccharide (LPS) covalently linked to cholera enterotoxin (CT), has also been prepared (22) but has not yet been evaluated in humans. A potential drawback is that the presence of free toxin remaining after the conjugation step necessitates additional purification and introduces some element of risk. Although CT is essential for the life-threatening diarrhea of cholera, vaccines consisting of chemically inactivated CT administered parenterally, or of its immunodominant B-subunit protein administered perorally with killed vibrios, were minimally protective; in the latter instance, the results of an extensive field study suggested that the addition of the B subunit actually reduced the efficacy of the whole-cell vaccine (11). The B-subunit protein, by itself, has previously been shown to be a less effective immunogen than other forms of CT antigen, including the holotoxin itself (13, 14). However, the disease cholera is itself an effective immunizing process in which both antitoxic and antibacterial antibodies are produced locally and systemically (27), having been stimulated by the in vivo-grown vibrios and their products. It would thus appear that the most effective way to induce immunity would be to simulate the disease process without evoking its undesirable symptoms, i.e., by use of a live attenuated vaccine.

CT is a multimeric protein exotoxin. The A ("active") protomer and the pentameric B ("binding") subunits are encoded by a single transcriptional unit, *ctxAB* (36). When posttranslationally modified by proteolysis and reduction (21), the catalytic A_1 subunit of CT is capable of ADP-ribosylating

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the guanine nucleotide-binding protein, which regulates cell adenylyl cyclase, leading to the severe diarrhea of cholera. The catalytic subunit of several ADP-ribosylating bacterial toxins share certain small regions of amino acid sequence identity (20, 41). Arg-9 is essential for enzymatic activity of pertussis toxin, and substitution of lysine for Arg-9 in the S-1 subunit of pertussis toxin abolishes ADP-ribosyltransferase activity and toxicity without altering a neutralizing epitope (8, 38). Similarly, the substitution of lysine for Arg-7 in the A subunit of CT, and in the A subunit of the CT-related heat-labile enterotoxin (LT) of *Escherichia coli*, eliminated ADP-ribosyltransferase activity (9, 29). Substitution of aspartic acid for Glu-110 or Glu-112 severely repressed ADP-ribosyltransferase activity of LT (29). Chemical mutagenesis that resulted in the substitution of lysine for Glu-112 caused a loss of toxicity and ADP-ribosyltransferase activity (46, 47). The protein did, however, interact with ADP-ribosylation factor (ARF), the 20-kDa guanine nucleotide-binding protein that serves as a GTP-dependent allosteric activator of CT and LT (34). Jobling and Holmes (24) have recently reported a variety of site-directed mutations of CT-A that had marked effects on holotoxin assembly and toxicity. To our knowledge, no one has yet substituted mutagenized *ctxA* for the wild-type gene in *Vibrio cholerae*.

Employing site-directed mutagenesis, in the present study we have created mutant CT-A subunit genes with codon substitutions for both Arg-7 and Glu-112, changes that eliminated ADP-ribosyltransferase activity. Genes encoding inactive analogs of CT (CT-1* and CT-2*, representing the two major epitypes of CT [13, 14, 31, 39]) were inserted into the chromosome of wild-type *V. cholerae* strains, including both serogroup O1 Inaba and Ogawa serotypes of El Tor and classical biotypes and the O139 (synonym Bengal) serogroup currently epidemic in India and Bangladesh (10, 40). The resulting recombinant strains may have potential as living attenuated vaccines, and the CT analogs could themselves be useful in conjugate vaccines and in structure-function studies.

MATERIALS AND METHODS

Bacterial strains, plasmids, media, and culture conditions. The bacterial strains and plasmids utilized are described in Table 1. The *E. coli* cells were grown at 37°C in Luria-Bertani broth medium (43). Antibiotics (Sigma Chemical Co., St. Louis, Mo.) were added at the following concentrations (in micrograms per milliliter): ampicillin, 50; kanamycin, 50; chloramphenicol, 10; tetracycline, 12.5 (*E. coli*) or 5 (*V. cholerae*). *V. cholerae* was cultured in syncase broth (15) for electroporation or toxin production.

DNA preparation, manipulation, and analysis. Plasmid DNA was extracted from *E. coli* DH5α cells by the alkaline lysis method of Birnboim and Doly (3). *V. cholerae* chromosomal DNA was prepared as described previously (2). Standard techniques (43) were used in the construction of recombinant plasmids. Restriction enzymes were generally obtained from Promega (Madison, Wis.), and digestions were done with buffers provided by the suppliers under the recommended conditions. Restriction fragments were electrophoresed in horizontal 0.8% agarose gels in TAE buffer (43) and stained with ethidium bromide (0.5 μg/ml). Restriction fragments were isolated from low-melting-point agarose (FMC BioProducts, Rockland, Maine) gels, melted at 55°C, and used directly in ligation reactions. Site-directed mutagenesis was performed as described previously (9).

Southern blot transfers were performed in 20× SSC (3 M NaCl, 0.3 M sodium citrate [pH 7.0]) with MagnaGraph nylon membranes (MSI, Westboro, Mass.). The probe, p3083 (5), was labelled with digoxigenin-11-dUTP by the random-primer method employed in the Genius System Kit (Boehringer Mannheim, Indianapolis, Ind.). Hybridizations were performed overnight at 55°C, and the membranes were washed at 55°C with a final stringency of 0.1× SSC plus 0.1% sodium dodecyl sulfate (SDS). Hybridizing bands were visualized by using the reagents and protocol for colorimetric detection provided in the Genius System Kit.

The *ctxAB* locus was amplified by PCR with the oligonucleotide primers 5′-dCTGTTAAACAAAGGGAGC-3′ (*ctxA*) and 5′-dCGGTTGCTTCTCATCATCG3′ (*ctxB*), using approximately 0.5 μg of chromosomal DNA and employing the reagents and protocol of the GeneAmp kit (Perkin Elmer Cetus, Norwalk, Conn.). Thirty cycles of amplification were carried out in a Perkin Elmer DNA Thermocycler with strand denaturation (1 min at 96°C), annealing (1.5 min at 49°C), and extension (1.5 min at 72°C). The amplified DNA products were cloned directly into the *Hin*cII site of pGEM3Zf(+).

Sequence analyses were carried out on an Applied Biosystems automated sequencer using dye terminators. The cloned PCR products were sequenced by dideoxy-chain termination reactions (44) with [α-^{35}S]dATP (NEN Research Products) and the Sequenase kit (United States Biochemical Corp., Cleveland, Ohio). Synthetic oligonucleotide primers were synthesized by the University of Missouri DNA Core Facility or at the Amgen-Boulder facility.

Electroporation. The *V. cholerae* strains were transformed by electroporation, with slight modifications of a published procedure (32). Bacterial cells were grown to mid-log phase in 20 ml of syncase medium (15) at 37°C, harvested and washed three times in the electroporation buffer (272 mM sucrose, 7 mM Na_2HPO_4 [pH 7.4], 1 mM $MgCl_2$), and resuspended in 1 ml of the same buffer. After the plasmid DNA was added, the bacteria were subjected to electric shock (Bio-Rad Gene Pulser, 25-μF capacitance, 2.5 kV) and allowed to recover in 1.5 ml of syncase medium at 37°C. The bacteria were pelleted, resuspended in 200 μl of medium, and plated on meat extract agar plates containing the appropriate antibiotics.

Recombinant CT analog production. For CT* production, 500-ml flasks containing 100 ml of syncase medium supplemented with antibiotics were inoculated with 2×10^6 viable bacteria per ml and incubated overnight (20 h) at 30°C with shaking. The recombinant toxin analogs were isolated from culture supernatants and partially purified by $Al(OH)_3$ adsorption and elution (18).

ARF-stimulated NAD:agmatine ADP-ribosyltransferase activity. The NAD:agmatine ADP-ribosyltransferase assay was performed essentially as described previously (34). Four micrograms of partially purified recombinant toxins was incubated at 30°C for 10 min in 70 mM glycine–30 mM dithiothreitol, pH 8.0. Wild-type CT holotoxin was treated similarly. Assay mixtures contained 50 mM potassium phosphate, pH 7.5, 5 mM $MgCl_2$, 100 μM GTP, 20 mM dithiothreitol, 0.1 mg of ovalbumin per ml, 100 μM [adenine-U-^{14}C]NAD (approximately 100,000 cpm), 3 mM dimyristoylphosphatidylcholine, 0.2% cholate, 10 mM agmatine, 0.5 μg of recombinant ARF 6, and 0.5 μg of wild-type or recombinant toxin in a 150-μl total volume. After incubation for 1 h at 30°C, 50-μl samples were transferred to columns of AG1-X2, which were washed five times with 1 ml of water each time. All eluates were collected for radioaassay by liquid scintillation counting.

Toxicity. Toxicity was assayed in vitro using Y-1 adrenal tumor cells (42) essentially as described previously (31). Samples containing up to 100 ng of toxin antigen (as determined by a radial diffusion immunoassay [19]) were added in duplicate

TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Description	Source (reference)[a]
E. coli DH5α	Host for recombinant plasmids	BRL
V. cholerae		
JBK70	*ctxAB* deletion derivative of El Tor, Inaba N16961	J. B. Kaper (28a)
CVD101	CT-A$^-$ CT-B$^+$ deletion derivative of classical, Ogawa 395	J. B. Kaper (28a)
CVD101/CT-1*	*ctxA***B-1*, derivative of CVD101, Kan^r	This study
CVD101/CT-2*	*ctxA***B-2*, derivative of CVD101, Kan^r	This study
CVD103	CT-A$^-$ CT-B$^+$ deletion derivative of classical, Inaba 569B	J. B. Kaper (28)
CVD103/CT-1*	*ctxA***B-1*, derivative of CVD103, Kan^r	This study
CVD103/CT-2*	*ctxA***B-2*, derivative of CVD103, Kan^r	This study
C6706	Wild-type El Tor, Inaba, from Peru, 1991	K. Wachsmuth, CDC
C6706/CT-1*	*ctxA***B-1*, *zot* mutant derivative of C6706, Kan^r	This study
C6706/CT-2*	*ctxA***B-2*, *zot* mutant derivative of C6706, Kan^r	This study
C7258	Wild-type El Tor, Ogawa, from Peru, 1991	K. Wachsmuth, CDC
C7258/CT-1*	*ctxA***B-1*, *zot* mutant derivative of C7258, Kan^r	This study
C7258/CT-2*	*ctxA***B-2*, *zot* mutant derivative of C7258, Kan^r	This study
SG25-1	Wild-type, O139 (synonym Bengal), from India, 1993	G. B. Nair (40)
SG25-1/CT-1*	*ctxA***B-1*, *zot* mutant derivative of SG25-1, Kan^r	This study
SG25-1/CT-2*	*ctxA***B-2*, *zot* mutant derivative of SG25-1, Kan^r	This study
Plasmids		
p3083	8.3-kb *Pst*I fragment containing *ctxAB-2* from *V. cholerae* 3083 cloned into pBR328; Cm^r Tet^r	T. J. Brickman (5)
pJM17	5.1-kb *Eco*RI-*Pst*I fragment containing *ctxAB-1* from *V. cholerae* 569B cloned into pBR322; Tet^r	J. J. Mekalanos (36)
pCT-A*	1.0-kb *Eco*RI-*Hin*cII fragment containing *ctxA** with Arg-7→Lys (CGG→AAG) and Glu-112→Gln (GAA→CAG) changes in pUC19; Amp^r	This study
p3083*	Derivative of p3083 in which the 0.8-kb *Nde*I fragment has been replaced by the analogous *Nde*I fragment from pCT-A*; Cm^r Tet^r, CT-2*	This study
pCT-2*	Derivative of p3083* with a Kan^r gene inserted into the *Nsi*I site within *zot*; Cm^r Tet^r Kan^r CT-2*	This study
p3083*-BstXI	Derivative of p3083* in which the 0.75-kb *Bst*XI fragment has been replaced by the analogous *Bst*XI fragment from pJM17; Cm^r Tet^r CT-1*	This study
pCT-1*	Derivative of p3083*-*Bst*XI with the Kan^r gene inserted into the *Nsi*I site within *zot*; Cm^r Tet^r Kan^r CT-1*	This study
pGEM3Zf(+)	Amp^r *lacZ*α	Promega
pUC19	Amp^r *lacZ*α	BRL
p657	Amp^r subclone of pBR322, a derivative of ColE1, with 1 kb of rickettsial DNA inserted into *Hin*dIII	G. A. McDonald, unpublished

[a] BRL, Bethesda Research Laboratories; CDC, Centers for Disease Control and Prevention.

to microtiter wells containing cultured Y-1 cells. CT-1 and CT-2 (1 ng each) were used as positive controls, and the results were recorded after 6 h of incubation.

Enterotoxicity. The ability of recombinant toxin analogs to cause diarrhea, and the virulence of strains producing CT-1* and CT-2*, were assayed in infant rabbits by standard methods employed in our laboratory (16): toxin and toxin analogs were fed to rabbits in 5 ml of 1% Tris, pH 8.0, whereas the bacterial strains were inoculated intraintestinally. Choleragenic scores were calculated for experimental groups of three or more animals.

Antigens and antibodies. CT-1 and CT-2, used as standards, were purified in our laboratory from fermentor-grown cultures (18) of classical biotype, Inaba serotype *V. cholerae* strain 569B and El Tor biotype, Ogawa serotype strain 3083 T, respectively. Hyperimmune rabbit polyclonal antisera used in this study were previously raised in this laboratory against CT-1 A- and B-subunit proteins and against CT-2 B-subunit protein. Unlike the B-subunit proteins, the A subunits of the two biotypes are identical (12). Monoclonal antibodies (MAbs) against B-subunit proteins used in this study were described previously and included anti-CT-1 (α-CT-1) and α-CT-2 classes I, III, and VI, α-H-LT class II, α-pDL-3 class IV, and α-S-LT class V (26). MAbs KB9 and 8E11 against the A subunit of CT-1 were also used (17).

Microzone electrophoresis. The recombinant proteins were analyzed by using the Microzone cellulose acetate electrophoresis system (Beckman Instruments Inc., Fullerton, Calif.) (6).

SDS-PAGE and Western blotting (immunoblotting). Samples of proteins were subjected to polyacrylamide gel electrophoresis (PAGE) in 4% stacking and 15% separating polyacrylamide gels and transferred onto nitrocellulose membranes as described previously (7).

G_{M1} binding. G_{M1} binding was evaluated by the Ouchterlony double diffusion in gel method. The checkerboard immunoblotting procedure (25), the G_{M1} modification (26), and the G_{M1} enzyme-linked immunosorbent assay (G_{M1}-ELISA) have been described (17).

RESULTS

Construction of plasmids encoding CT-2* and CT-1*. Plasmid pCT-A* was constructed by subcloning a synthetic oligonucleotide linker with an Arg-7→Lys codon substitution (CGG→AAG) into the mutated *ctxA* gene containing a Glu-112→Gln (GAA→CAG) mutation, thus combining both codon substitutions. An 8.3-kb *Pst*I fragment carrying the *ctxAB* genes from *V. cholerae* 3083, a CT-2 producer, was previously cloned into pBR328 (p3083) (5). To place the mutated CT-A subunit gene in a functional genetic environment for toxin production, the wild-type *ctxA* gene of p3083 was replaced with the mutated *ctxA** gene by exchange of the analogous 0.8-kb *Nde*I restriction fragment containing most of the CT-A subunit gene, thus generating p3083*. The CT gene from *V. cholerae* 569B, which produces CT-1, was previously cloned into pBR322 (pJM17) (36). To combine the mutated *ctxA** gene with the gene encoding the B subunit of CT-1, the 0.75-kb *Bst*XI fragment of pJM17, containing the majority of *ctxB-1*, was used to replace the *Bst*XI fragment of p3083*. The presence of both codon mutations in plasmids p3083* and p3083*-BstXI, now encoding the toxin analogs CT-2* and CT-1*, respectively, was confirmed by DNA sequencing. Further, a Kan^r gene cassette was inserted into the *Nsi*I site in the *zot* gene (1), which is located immediately upstream of *ctxA*B*, in p3083* and p3083*-BstXI, thus generating pCT-2* and pCT-1*, respectively.

Construction of *V. cholerae* strains carrying the inactive toxin gene. Plasmids pCT-1* and pCT-2* were introduced into wild-type El Tor biotype *V. cholerae* C6706 and C7258, O139 strain SG25-1, and mutant classical vibrio strains CVD101 and CVD103 by electroporation. Strains carrying the plasmids were then transformed by a second, incompatible plasmid, p657, and selected for Amp resistance. Kan^r vibrios that had lost all plasmid resistance markers (and thus should carry the Kan^r cassette in the chromosomal *zot* gene after a homologous recombination event) were isolated that showed no toxin activity in the Y1 assay. Southern blot analysis of restriction enzyme-digested chromosomal DNA of the recombinant strains and the wild-type parents demonstrated the expected larger size of the restriction fragments carrying the CT gene region in the mutant strains (Fig. 1). Interestingly, SG25-1 wild-type DNA differs from that of the Peru isolates in both the *Pst*I and *Hin*dIII digests, consistent with another report suggesting an amplification of the CT gene cassette in the O139 strains (48). However, the mutant strains created here show band patterns similar to those of the mutant strains created from the Peru isolates, indicating the deletion of all additional copies of CT genes. Since the Kan^r gene cassette introduces a *Hin*dIII site in the mutant DNA that is not present in the parental DNA, an additional band is observed in those lanes (Fig. 1). To confirm the presence of both point mutations in the *ctxA* genes and the successful recombination of the *ctxB* genes encoding the B subunit of CT-1 in C6706/CT-1*, C7258/CT-1*, and SG25-1/CT-1*, the entire *ctxAB* operon was amplified by PCR from both the mutant and parental wild-type strains; DNA fragments of approximately 1.2 kb were amplified, cloned, and partially sequenced. In all mutant strains, codon substitutions for both Arg-7 and Glu-112 were present in the *ctxA** genes. As expected, differences in nucleotide sequences between the *ctxB* genes encoding the B subunits of CT-1 and CT-2 were found: the ctxB genes from C6706/CT-1* C7258/CT-1*, and SG25-1/CT-1* encode His-18 and Thr-47 (CT-1), whereas those of C6706/CT-2*, C7258/CT-2*, and SG25-1/CT-2* encode Tyr-18 and Ile-47 (CT-2) (12, 39).

Toxin analog characterization. Strain JBK70 was transformed with plasmids pCT-1* and pCT-2* for the purpose of producing toxin analogs for analysis. The yields of analog proteins from plasmid-carrying strain JBK70 ranged from approximately 0.15 to 0.25 μg/ml. Examination by Western blotting of the toxin analogs, CT-1* and CT-2*, partially purified with $Al(OH)_3$, revealed unnicked A* (28 kDa), A_1* (21 kDa), and B (11.5 kDa) protein bands (Fig. 2). Because CT-1*, CT-2, and CT-2* were rapidly isolated, the A subunit

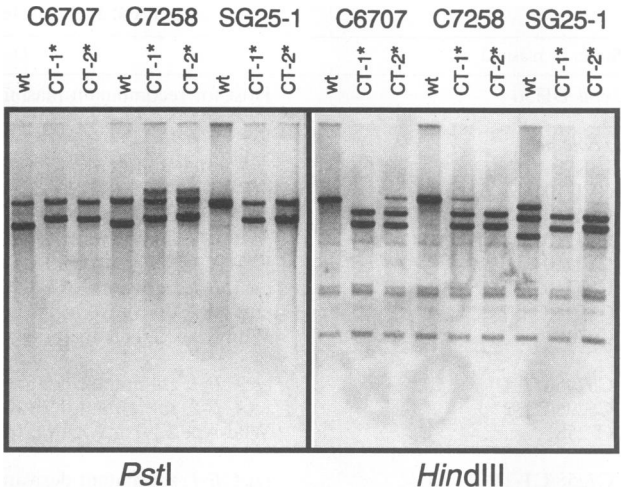

FIG. 1. Southern blot analysis, using p3083 as a probe, of chromosomal DNA from *V. cholerae* wild-type (wt) strains and their derivatives encoding recombinant toxins (CT-1* or CT-2*) digested with restriction enzymes *Pst*I and *Hin*dIII. The *Pst*I digest reveals that the lower (smaller) band increases by approximately 1.3 kb due to the insertion of a Kan^r cassette in *zot*. SG25-1, an O139 serotype strain, differs from the El Tor biotype O1 strains from Peru, C6707 and C7258, but assumes the same pattern after recombination. The insertion of the Kan^r cassette introduces an additional *Hin*dIII site. SG25-1 again differs from the El Tor biotype strains and assumes the same pattern after recombination.

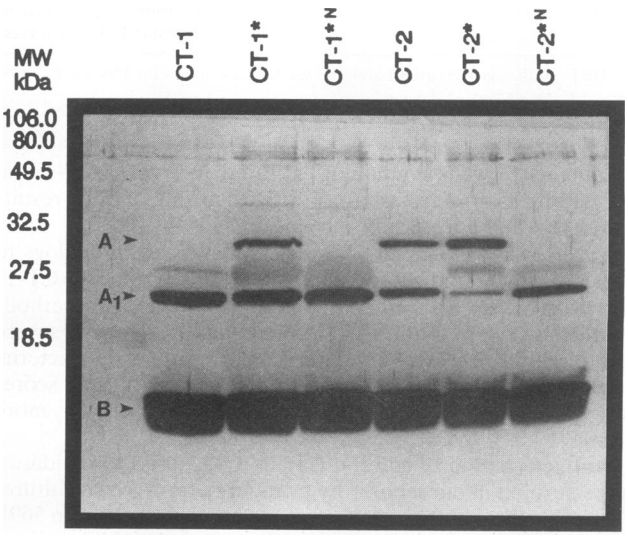

FIG. 2. Western blot analysis of partially purified CT analogs. The A-subunit proteins of CT-1*, CT-2, and CT-2*, prepared rapidly, were largely unnicked. CT-1*^{N} and CT-2*^{N} were pretreated with trypsin to form CT-A_1*. The blots were developed using specific polyclonal rabbit α-A and α-B (1:1,000) prepared in this laboratory.

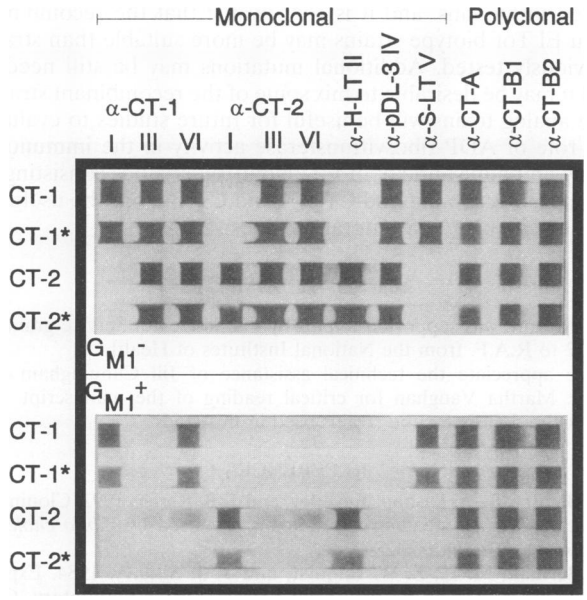

FIG. 3. Checkerboard immunoblotting analysis of the reactivity of CT and CT* with monoclonal and polyclonal antibodies and the effect of G_{M1}. α-CT-1 class I recognizes only CT-1 and CT-1*. α-CT-1 class III recognizes both CT-1 and CT-2 and their derivatives but is blocked by G_{M1}. α-CT-1 class VI recognizes both epitypes, but its reactivity with CT-2 is blocked by G_{M1}. α-CT-2 class I recognizes only CT-2 and its derivative. α-CT-2 class III and class VI recognize both epitypes and are blocked by G_{M1}. α-H-LT class II recognizes only CT-2 and CT-2*. α-pDL-3 class IV recognizes both epitypes and is blocked by G_{M1}. α-S-LT class V recognizes only CT-1 and CT-1*. Polyclonal antibodies react with both epitypes and their derivatives similarly.

was predominantly unnicked (4). When the toxin analogs were treated overnight at 24°C with 1.8 μg of typsin per ml (21), they were fully converted to a nicked form (Fig. 2).

The partially purified toxin analogs migrated identically to wild-type CT in cellulose acetate membrane electrophoresis, and the single protein bands were stained with both α-A and α-B polyclonal antibodies. To determine whether the amino acid substitutions affected the immunological properties of these toxin analogs, antitoxin MAbs (26) were used to compare the reactivity of the CT* proteins with wild-type toxins. The reactions of the toxins and their analogs with various antibodies in checkerboard immunoblotting are shown in Fig. 3. In contrast to CT-2 and CT-2*, CT-1 and CT-1* are recognized by α-CT-1 class I and α-S-LT class V MAbs but are not recognized by α-CT-2 class I, and α-H-LT class II. The reactions of α-CT-1 class III, α-CT-2 class III and VI, and α-pDL-3 class IV MAbs with both CT epitypes and their derivatives were blocked by G_{M1} (Fig. 3). However, the reaction of α-CT-1 class VI MAb was blocked by G_{M1} only with CT-2 and CT-2*. Polyclonal antibodies recognized mutant and wild-type proteins equally well. Thus, the immunological properties of the CT analogs, by these criteria, were identical to those of wild-type toxins.

The ability of the B subunits of the toxin analogs to recognize and react with G_{M1} ganglioside was further examined by G_{M1} immunodiffusion-in-gel analysis (G_{M1}-Ouchterlony). Recombinant toxin analogs formed precipitation bands with G_{M1} ganglioside that were indistinguishable from those of wild-type CTs. Results of G_{M1}-ELISA indicated that the toxin analogs formed intact heteropolymers. The toxin analogs reacted well with polyclonal α-A and polyclonal α-B sera in this assay, showing that, in binding to G_{M1}, the B pentamer carried the A subunit protein with it. However, the reactions of α-A subunit MAbs with both CT-1* and CT-2* were weaker than those with CT-1 and CT-2 standards: i.e., the optical densities for the reactions between hybridoma supernatant (1:10) with approximately 100 ng of analog antigen ranged from 1.4- to 4-fold lower than those with the standards (e.g., 0.16 to 0.3 versus 0.43 to 0.65).

The purified toxin analogs, at up to 100-fold higher concentrations, failed to elicit the typical rounding of cultured Y-1 adrenal tumor cells that was observed with wild-type toxins. Additionally, the toxin analogs possessed no NAD:agmatine ADP-ribosyltransferase activity in the presence of ARF. Infant rabbits infected with 10^6 to 10^7 wild-type live vibrios or fed 5 μg of CT showed typical ante mortem symptoms or died of diarrhea. Significantly, none of the rabbits infected with recombinant *V. cholerae* strains producing CT* or fed 25 μg of CT* had diarrhea or fluid accumulation in the small intestines (choleragenic score, <1). *V. cholerae* organisms were recovered in practically pure culture from the intestines of the rabbits challenged with wild-type and recombinant strains.

DISCUSSION

The ultimate goal of this work is the development of a cholera vaccine which is effective (preferably in a single dose), economical, convenient, and suitably nonreactogenic. For reasons which have been discussed elsewhere (13, 14, 27), none of the many candidate vaccines which have been developed in the past has met all of these criteria; they have been insufficiently potent, too reactogenic, too expensive and inconvenient, or exhibited a combination of these deficiencies. One promising vaccine candidate, CVD103-HgR (a CT-A^- CT-B^+ mutant of classical Inaba strain 569B), is well tolerated but, like its parent strain, poorly colonizes the gut; high doses are required (33), and it has a diminished protective efficacy against the more prevalent El Tor biotype (28). This reduced effectiveness against the El Tor biotype is yet unexplained but could relate to the fact that it is an A^- B^+ strain (i.e., it has no CT-A antigen); further, the B-subunit protein it produces is of the CT-1 epitype, whereas most El Tor vibrios (except the Gulf Coast clone [personal observations]) produce CT-2. In addition, CVD103-HgR does not produce the cell-associated mannose-sensitive hemagglutinin that is characteristic of El Tor biotype strains (23) and, because of somatic antigen differences, it is likely to be ineffective against the new O139 serogroup strains.

It is not clear why CT antigens containing A-subunit protein (e.g., the holotoxin or procholeragenoid) elicit stronger immune responses than the B-subunit pentamer (choleragenoid) (37). The fact that procholeragenoid (a heat-induced aggregate of CT) was minimally toxic, but equally as immunogenic as the holotoxin, would suggest that ADP-ribosyltransferase activity was not responsible for its enhanced immunogenicity. However, these observations are complicated by its high molecular weight and slight residual CT-like activity. CT has been recognized since the pioneer study of Northrup and Fauci (35) to be a powerful immunodulator, having both adjuvant and immunosuppressive effects that are dependent on dose and time of administration. Lycke et al. (30) have recently reported that analog LT (with lysine substituted for Glu-112), lacking ADP-ribosyltransferase activity, like CT-B but unlike CT or LT, had no adjuvant activity for keyhole limpet hemocyanin. These investigators concluded that adjuvanticity was directly linked to ADP-ribosyltransferase activity, but since the amino acid substitution is rather nonconservative, altering the

pI of the protein (47), other explanations are clearly feasible. The immunogenicity of the analog and native proteins were not compared in the study by Lycke et al. (30).

In the present investigation, conservative substitutions for both Arg-7 and Glu-112, each of which have been shown to eliminate ADP-ribosyltransferase activity, were introduced as codon substitutions into the gene encoding the catalytic A subunit of CT. The mutated *ctxA** gene was joined with cloned *ctxB* genes from classical and El Tor biotype *V. cholerae* strains to produce the recombinant toxin analogs CT-1* and CT-2*, respectively. The toxin analogs were partially purified and found to lack (i) toxicity for cultured adrenal tumor cells, (ii) ADP-ribosyltransferase activity in vitro, and (iii) diarrheagenic activity (in infant rabbits). They retained their ability to form heteropolymers and to interact with G_{M1}, and they reacted similarly to the wild type with MAbs against CT-B. However, their reactivity with α-CT-A MAbs was weaker than that of the wild type, perhaps reflecting some conformational difference in an epitope or epitopes, recognized by the α-CT-A MAbs, which are dependent on Arg-7 and/or Glu-112.

The genes encoding the recombinant toxin analogs were introduced by homologous recombination into the chromosomes of El Tor biotype *V. cholerae* C6706 (Inaba) and C7258 (Ogawa), recently isolated in Peru; SG25-1, an O139 serotype strain from the current epidemic in India; and classical biotype mutant vibrios CVD101 and CVD103, CT-A^- CT-B^+ derivatives of strains 396 (Ogawa) and 569B (Inaba), respectively. Southern blot analyses indicated the insertion of the kanamycin gene in *zot*. Amplification of the CT genes by PCR and partial nucleotide sequencing confirmed the presence of both codon substitutions in the *ctxA** genes and the appropriate *ctxB* genes. Our observations do not as yet permit the likely conclusion that the original *ctxB-1* is retained in the classical biotype CT-2*-producing mutants; i.e., they could conceivably be producing chimeras of CT*-B-1 and CT*-B-2 which could be advantageous. Studies are in progress to enable us to make this determination.

The El Tor and O139 recombinant strains were avirulent in the infant rabbit animal model. Whether they are suitably avirulent for use in humans can be determined only by trial in volunteers. Previous studies with small numbers of volunteers have demonstrated that Tox^- mutants (e.g., JBK70), although diarrheagenic by themselves, elicited significant protection against subsequent challenge with the virulent wild type (28a). The mechanisms of this protection are not clear, although an antibacterial (anti-LPS) component was presumed to be involved since the challenged volunteers yielded fewer vibrios on coproculture. Whether engineered strains expressing toxin analogs containing both A and B subunits would provide better and more durable homologous immunity remains to be tested. They could potentially also offer better cross-protection against related enterotoxins if A*B is more effective than B alone as an antigen and adjuvant. It is also evident from the recent volunteer studies with Tox^- mutants that, even though CT is the factor responsible for the severe, life-threatening diarrhea of cholera, the vibrios have other mechanisms for causing milder forms of diarrhea. CVD110, a mutant from El Tor biotype Ogawa serotype strain E7946 deleted for all known virulence factors, was still capable of causing diarrhea (mean stool volume of 861 ml) in 7 of 10 volunteers (45). The act of colonization may itself be diarrheagenic; alternatively, another, yet undiscovered, cholera enterotoxin may be responsible. As the capability of mutant *V. cholerae* strains to cause diarrhea is still an unpredictable event, volunteer studies with the mutants described herein may provide useful information. For example, derivatives of serogroup O139 have yet to be tested in humans, and it is conceivable that the recombinant Peru El Tor biotype strains may be more suitable than strains previously tested. Additional mutations may be still needed, and it may be desirable to mix some of the recombinant strains. The analog toxins will be useful for future studies to evaluate the role of ADP-ribosyltransferase activity in the immunogenicity and adjuvanticity of CT. Nontoxic analogs consisting of an altered A subunit and CT-B-1 and CT-B-2 may be useful as oral or conjugate parenteral vaccines (22).

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REFERENCES

1. **Baudry, B., A. Fasano, J. 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. **Berman, M. L., L. W. Enquist, and T. J. Silhavy.** 1984. Experiments with gene fusions. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
3. **Birnboim, H. C., and J. Doly.** 1979. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Res. **7**:1513–1523.
4. **Booth, B. A., M. Boesman-Finkelstein, and R. A. Finkelstein.** 1984. *Vibrio cholerae* hemagglutinin/protease nicks cholera enterotoxin. Infect. Immun. **45**:558–560.
5. **Brickman, T. J., M. Boesman-Finkelstein, R. A. Finkelstein, and M. A. McIntosh.** 1990. Molecular cloning and nucleotide sequence analysis of cholera toxin genes of the $CtxA^-$ *Vibrio cholerae* strain Texas Star-SR. Infect. Immun. **58**:4142–4144.
6. **Briere, R. O., and J. D. Mull.** 1964. Electrophoresis of serum proteins with cellulose acetate. Am. J. Clin. Pathol. **42**:547–551.
7. **Burnette, W. N.** 1981. "Western blotting". electrophoretic transfer of proteins from sodium dodecyl sulfate polyacrylamide gels to unmodified nitrocellulose and radiographic detection with antibody and radioiodinated protein A. Anal. Biochem. **112**:195–203.
8. **Burnette, W. N., W. Cieplak, V. L. Mar, K. T. Kaljot, H. Sato, and N. M. Keith.** 1988. Pertussis toxin S1 mutant with reduced enzyme activity and a conserved protective epitope. Science **242**:72–74.
9. **Burnette, W. N., V. L. Mar, B. W. Platler, J. D. Schlotterbeck, M. D. McGinley, K. S. Stoney, M. F. Rohde, and H. R. Kaslow.** 1991. Site-specific mutagenesis of the catalytic subunit of cholera toxin: substituting lysine for arginine 7 causes loss of activity. Infect. Immun. **59**:4266–4270.
10. **Cholera Working Group.** 1993. Large epidemic of cholera-like disease in Bangladesh caused by *Vibrio cholerae* O139 synonym Bengal. Lancet **342**:387–390.
11. **Clemens, J. D., D. A. Sack, J. R. Harris, F. van Loon, J. Chakraborty, F. Ahmed, M. R. Rao, M. R. Khan, M. Yunus, N. Huda, B. F. Stanton, B. A. Kay, S. Walter, R. Eeckels, A.-M. Svennerholm, and J. Holmgren.** 1990. Field trial of cholera vaccines in Bangladesh: results from three-year follow-up. Lancet **335**:270–273.
12. **Dams, E., M. de Wolf, and W. Dierick.** 1991. Nucleotide sequence analysis of the CT operon of the *Vibrio cholerae* classical strain 569B. Biochim. Biophys. Acta **1090**:139–141.
13. **Finkelstein, R. A.** 1992. Cholera enterotoxin (choleragen): a historical perspective, p. 155–187. *In* D. Barua and W. B. Greenough III (ed.), Current topics in infectious diseases: Cholera. Plenum Medical Publishers, New York.
14. **Finkelstein, R. A.** 1993. Why do we not yet have a safe, effective and economical vaccine against cholera?, p. 137–153. *In* A. F. Pestana de Castro and W. F. Almeida (ed.), Cholera on the American continents. International Life Sciences Institute/ILSA Press, Washington, D.C.
15. **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.

16. **Finkelstein, R. A., M. Boesman-Finkelstein, Y. Chang, and C. C. Häse.** 1992. *Vibrio cholerae* hemagglutinin/protease, colonial variation, virulence, and detachment. Infect. Immun. **60:**472–478.
17. **Finkelstein, R. A., M. F. Burks, A. Zupan, W. S. Dallas, C. O. Jacob, and D. S. Ludwig.** 1987. Epitopes of the cholera family of enterotoxins. Rev. Infect. Dis. **9:**544–561.
18. **Finkelstein, R. A., K. Fujita, and J. J. LoSpalluto.** 1971. Procholeragenoid: an aggregated intermediate in the formation of choleragenoid. J. Immunol. **107:**1043–1051.
19. **Finkelstein, R. A., and J. J. LoSpalluto.** 1969. Pathogenesis of experimental cholera: preparation and isolation of choleragen and choleragenoid. J. Exp. Med. **130:**185–202.
20. **Gill, D. M.** 1988. Sequence homologies among the enzymatically active portions of ADP-ribosylating toxins. Zentralbl. Bakteriol. Suppl. **17:**315–323.
21. **Gill, D. M., and R. S. Rappaport.** 1979. Origin of the enzymatically active A1 fragment of cholera toxin. J. Infect. Dis. **139:**674–680.
22. **Gupta, R. K., S. C. Szu, R. A. Finkelstein, and J. B. Robbins.** 1992. Synthesis, characterization and some immunological properties of conjugates composed of the detoxified lipopolysaccharide of *Vibrio cholerae* O1 serotype Inaba bound to cholera toxin. Infect. Immun. **60:**3201–3208.
23. **Hanne, L. F., and R. A. Finkelstein.** 1982. Characterization and distribution of the hemagglutinins produced by *Vibrio cholerae*. Infect. Immun. **36:**209–214.
24. **Jobling, M. G., and R. K. Holmes.** 1993. Molecular genetic studies of the structure and function of the A subunit of cholera enterotoxin, p. 83–87. *In* Proceedings of the 29th Joint Conference, U.S.-Japan Cooperative Medical Science Program, Cholera and Related Diarrheal Diseases. National Institutes of Health, Bethesda, Md.
25. **Kazemi, M., and R. A. Finkelstein.** 1990. Checkerboard immunoblotting (CBIB): an efficient, rapid, and sensitive method of assaying multiple antigen/antibody cross-reactivities. J. Immunol. Methods **128:**143–146.
26. **Kazemi, M., and R. A. Finkelstein.** 1990. Study of epitopes of cholera enterotoxin-related enterotoxins by checkerboard immunoblotting. Infect. Immun. **58:**2352–2360.
27. **Levine, M. M., J. B. Kaper, R. E. Black, and M. L. Clements.** 1983. New knowledge on pathogenesis of bacterial enteric infections as applied to vaccine development. Microbiol. Rev. **47:**510–550.
28. **Levine, M. M., J. B. Kaper, D. Herrington, J. Ketley, G. A. Losonsky, C. O. Tacket, B. D. Tall, and S. J. Cryz.** 1988. Safety, immunogenicity and efficacy of recombinant live oral cholera vaccines, CVD103 and CVD103-HgR. Lancet **II:**467–470.

28a. **Levine, M. M., J. B. Kaper, D. Herrington, G. Losonsky, J. G. Morris, M. L. Clements, R. E. Black, B. Tall, and R. Hall.** 1988. Volunteer studies of deletion mutants of *Vibrio cholerae* O1 prepared by recombinant techniques. Infect. Immun. **56:**161–167.

29. **Lobet, Y., C. W. Cluff, and W. Cieplak, Jr.** 1991. Effect of site-directed mutagenic alterations on ADP-ribosyltransferase activity of the A subunit of *Escherichia coli* heat-labile enterotoxin. Infect. Immun. **59:**2870–2879.
30. **Lycke, N., T. Tsuji, and J. Holmgren.** 1992. The adjuvant effect of *Vibrio cholerae* and *Escherichia coli* heat-labile enterotoxins is linked to their ADP-ribosyltransferase activity. Eur. J. Immunol. **22:**2277–2281.
31. **Marchlewicz, B. A., and R. A. Finkelstein.** 1983. Immunologic differences among the cholera/coli family of enterotoxins. Diagn. Microbiol. Infect. Dis. **1:**129–138.
32. **Marcus, H., J. M. Ketley, J. B. Kaper, and R. K. Holmes.** 1990. Effects of DNase production, plasmid size, and restriction barriers on transformation of *Vibrio cholerae* by electroporation and osmotic shock. FEMS Microbiol. Lett. **68:**149–154.
33. **Migasena, S., P. Pitisuttitham, B. Prayurahong, P. Suntharasamai, W. Supanaranond, V. Desakorn, U. Vongsthongsri, B. Tall, J. Ketley, G. Losonsky, S. Cryz, J. B. Kaper, and M. M. Levine.** 1989. Preliminary assessment of the safety and immunogenicity of live oral cholera vaccine strain CVD103-HgR in healthy Thai adults. Infect. Immun. **57:**3261–3264.
34. **Moss, J., S. J. Stanley, M. Vaughan, and T. Tsuji.** 1993. Interaction of ADP-ribosylation factor with *Escherichia coli* enterotoxin that contains an inactivating lysine 112 substitution. J. Biol. Chem. **268:**6383–6387.
35. **Northrup, R. S., and A. S. Fauci.** 1972. Adjuvant effect of cholera enterotoxin on the immune response of the mouse to sheep red blood cells. J. Infect. Dis. **125:**672–673.
36. **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.
37. **Pierce, N. F., W. C. Cray, Jr., J. B. Sacci, Jr., J. P. Craig, R. Germanier, and E. Führer.** 1983. Procholeragenoid: a safe and effective antigen for oral immunization against experimental cholera. Infect. Immun. **40:**1112–1118.
38. **Pizza, M., A. Covacci, A. Bartoloni, M. Perugini, L. Nencioni, M. T. De Magistris, L. Villa, D. Nucci, R. Manetti, M. Bugnoli, F. Giovannoni, R. Olivieri, J. T. Barbieri, H. Sato, and R. Rappuoli.** 1989. Mutants of pertussis toxin suitable for vaccine development. Science **246:**497–500.
39. **Qu, Z.-H., and R. A. Finkelstein.** 1993. Checkerboard immunoblotting recognizes twenty epitopes among the B subunit proteins of the cholera enterotoxin family. Electrophoresis **14:**899–901.
40. **Ramamurthy, T., S. Garg, R. Sharma, S. K. Bhattacharya, G. B. Nair, T. Shimada, T. Takeda, T. Karasowa, H. Kurazono, A. Pal, and Y. Takeda.** 1993. Emergence of novel strain of *Vibrio cholerae* with epidemic potential in southern and eastern India. Lancet **341:**703–704.
41. **Rappuoli, R., and M. Pizza.** 1991. Structure and evolutionary aspects of ADP-ribosylating toxins, p. 1–21. *In* J. E. Alouf and J. H. Freer (ed.), Sourcebook of bacterial protein toxins. Academic Press, Inc., San Diego, Calif.
42. **Sack, D. A., and R. B. Sack.** 1974. Test for enterotoxigenic *Escherichia coli* using Y-1 adrenal cells in miniculture. Infect. Immun. **11:**334–336.
43. **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.
44. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74:**5463–5467.
45. **Tacket, C. O., G. Losonsky, J. P. Nataro, S. J. Cryz, R. Edelman, A. Fasano, J. Michalski, J. B. Kaper, and M. M. Levine.** 1993. Safety and immunogenicity of live oral cholera vaccine candidate CVD110, a Δ*ctxA* Δ*zot* Δ*ace* derivative of El Tor Ogawa *Vibrio cholerae*. J. Infect. Dis. **168:**1536–1540.
46. **Tsuji, T., T. Inoue, A. Miyama, and N. Noda.** 1991. Glutamic acid-112 of the A subunit of heat-labile enterotoxin from enterotoxigenic *Escherichia coli* is important for ADP-ribosyltransferase activity. FEBS Lett. **291:**319–321.
47. **Tsuji, T., T. Inoue, A. Miyama, K. Okamoto, T. Honda, and T. Miwatani.** 1990. A single amino acid substitution in the A subunit of *Escherichia coli* enterotoxin results in a loss of its toxic activity. J. Biol. Chem. **36:**22520–22525.
48. **Waldor, M. K., and J. J. Mekalanos.** 1993. Molecular analysis of virulence determinants carried by the "Bengal" strain of *Vibrio cholerae* O139 and construction of vaccine prototypes, p. 42–46. *In* Proceedings of the 29th Joint Conference, U.S.-Japan Cooperative Medical Science Program, Cholera and Related Diarrheal Diseases. National Institutes of Health, Bethesda, Md.