INFECTION AND IMMUNITY, Oct. 1993, p. 4462–4468
0019-9567/93/104462-07$02.00/0

Vol. 61, No. 10

CVD110, an Attenuated *Vibrio cholerae* O1 El Tor Live Oral Vaccine Strain

JANE MICHALSKI,[1] JAMES E. GALEN,[1] ALESSIO FASANO,[2] AND JAMES B. KAPER[1]*

Center for Vaccine Development, Division of Geographic Medicine, Department of Medicine,[1] and Division of Pediatric Gastroenterology and Nutrition, Department of Pediatrics,[2] University of Maryland School of Medicine, 10 S. Pine Street, Baltimore, Maryland 21201

Received 23 March 1993/Returned for modification 3 May 1993/Accepted 27 July 1993

The recent expansion of the seventh cholera pandemic into South America emphasizes the need for a safe, long-lasting, protective, and nonreactogenic vaccine for this disease. Since the predominant *Vibrio cholerae* O1 strains in the world today are of the El Tor biotype, a bivalent vaccine containing both classical and El Tor biotypes may be desirable. We have constructed a new oral vaccine candidate, *V. cholerae* CVD110 El Tor, Ogawa, from which all toxin genes so far identified in *V. cholerae* have been deleted. Three of these genes, those encoding cholera toxin (*ctx*), zonula occludens toxin (*zot*), and accessory cholera enterotoxin (*ace*), are located on a 4.5-kb virulence cassette flanked by repetitive sequences (RS1 elements). Homologous recombination between these RS1 elements resulted in the deletion of this virulence cassette to yield *V. cholerae* CVD109. Insertion of genes encoding mercury resistance (*mer*) and the cholera toxin B subunit (*ctxB*) into the hemolysin locus (*hlyA*) produced CVD110. This insertion serves three purposes. (i) It genetically tags the vaccine strain so as to distinguish it from wild-type *V. cholerae* O1. (ii) It produces cholera toxin B subunit in order to elicit antitoxic immunity. (iii) It inactivates the hemolysin gene, rendering the strain nonhemolytic on sheep erythrocyte plates. Supernatants from *V. cholerae* CVD110 cultures are nonreactogenic when assayed in Ussing chambers.

Vibrio cholerae O1, the etiologic agent of cholera, causes acute diarrhea, leading to rapid and potentially fatal fluid and electrolyte loss. Since the early 1960s the seventh pandemic has spread from southeast Asia across the Middle East and into Africa and Central America (8). The recent introduction of cholera into South America (10, 33) has reemphasized the pressing need for a safe and effective vaccine against this disease.

Significant protective immunity can result from an initial infection with *V. cholerae*. Clinical volunteer studies conducted by Cash et al. (5) and Levine et al. (16, 19) have demonstrated that a single infection with a virulent *V. cholerae* classical or El Tor biotype confers 100 and 90% protection, respectively, against subsequent challenge with the homologous strain; this protection endures for at least 3 years (17). Several live, attenuated *V. cholerae* vaccine strains developed in our laboratory have been tested as live oral vaccine candidates in volunteer studies. From two recombinant vaccine candidates, JBK70 (El Tor, Inaba) and CVD101 (classical, Ogawa), genes encoding both the A and B subunits (JBK70) or the A subunit only (CVD101) of cholera toxin (CT) were deleted. These strains engendered high vibriocidal and antitoxic antibody responses in volunteers, and JBK70 conferred 89% protection against challenge with the wild-type El Tor Inaba strain N16961 (21).

Although the protective immunity of JBK70 and the high vibriocidal antibody titers elicited by both attenuated strains were encouraging, the utility of these strains is diminished by an unacceptable degree of reactogenicity (21). While the severe diarrhea produced by CT was not seen with these strains, many volunteers had a variety of symptoms, including mild diarrhea, low-grade fever, abdominal cramps, and anorexia. These side effects were hypothesized to be caused by additional toxins produced by *V. cholerae*. Recently, our laboratory has identified the genes encoding two new toxins, zonula occludens toxin (*zot*) (7) and accessory cholera enterotoxin (*ace*), which are located immediately upstream of the CT genes (*ctx*) within a 4.3-kb virulence cassette (34).

In contrast to JBK70 and CVD101, vaccine candidate CVD103-HgR (classical, Inaba) is well tolerated and confers a high level of protective immunity (82 to 100%) against homologous challenge with its parent strain, 569B (20, 32). Although CVD103-HgR gave 100% protection against severe cholera-like diarrhea in a heterologous challenge study using *V. cholerae* N16961 (El Tor, Inaba) and E7946 (El Tor, Ogawa), a reduced protective efficacy (62%) against any diarrhea was observed (20); i.e., some vaccinees developed mild diarrhea. Since the most prevalent biotype in the world today is El Tor (8, 10), it may be desirable to have an attenuated vaccine strain of the El Tor biotype which could be combined with CVD103-HgR in a bivalent vaccine. Here, we report a new, attenuated recombinant cholera vaccine candidate, *V. cholerae* CVD110 El Tor, Ogawa, from which all known toxin genes have been deleted.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. Relevant *Escherichia coli* strains, *V. cholerae* strains, and plasmids used in this study are listed in Table 1. Unless stated otherwise, all bacterial strains were propagated in L broth (26) or on L agar at 37°C, supplemented as needed with the following antibiotics or mercuric chloride: ampicillin (200 μg/ml), kanamycin (50 μg/ml), $HgCl_2$ (50 μM), and polymyxin B (50 U/ml) (Sigma Chemical Co., St. Louis, Mo.). AKI medium (1.5% Bacto Peptone, 0.4% yeast extract, 0.5% NaCl, 0.3% $NaCO_3$) (12) was used to culture *V. cholerae* to assay cholera B subunit production. SOC medium (4) (Life Technologies, Gaithersburg, Md.) was used to repair cells after electroporation and transformation procedures.

Molecular genetic techniques and reagents. Standard re-

* Corresponding author.

TABLE 1. Bacterial strains and plasmids

Bacterial strains and plasmids	Relevant genotype and/or phenotype	Reference
V. cholerae		
E7946	Wild-type El Tor, Ogawa	18
CVD109	Δ(*ctxAB zot ace*)	This study
CVD110	Δ(*ctxAB zot ace*) *hlyA*::(*ctxB mer*) Hg^r	This study
E. coli		
HB101	F^- *hsdS20* (r_B^- m_B^-) *recA13 leuB6 ara-14 proA2 lacY1 galK2 rpsL20* (Sm^r) *xly-5 mtl-1 supE44* λ^-	30
DH5α	F^- φ80d*lacZ*ΔM15 Δ(*lacZYA-argF*)*U169 endA1 recA1 hsdR17* (r_K^- m_K^+) *deoR thi-1 supE44 gyrA96 relA1* λ^-	4
SY327λ*pir*	Δ(*lac pro*) *argE*(Am) *rif nalA recA56* (λ*pir*R6K)	27
SM10λ*pir*	*thi thr leu tonA lacY supE recA*::RP4-2Tc::Mu (λ*pir*R6K) Km^r	27
Plasmids		
pBB168	pUC19::(*zot ctxB*)	3
pCVD30	pBR322::(*ace zot ctxB*)	13
pGP704	*mob*RP4 *ori*R6K Ap^r	27
pJMK12	*hlyA*::*mer ori*R6K Hly^- Hg^r Ap^r	15
pCVD315	pKO1 derivative, Ap^r	9
pCVD621	pCVD315::*ctxB*	This study
pCVD622.2B	pJMK12 derivative containing *hlyA*::(*ctxB mer*) *ori*R6K Hg^r Ap^r Hly^-	This study
pCVA2	pGP704::(*zot ctxB*) *ori*R6K Ap^r	This study
pDB8	pBR322::*mer* Hg^r	2
pCVD43	pRK290::Δ*hlyA*	14
pHC79	Ap^r	11
pRK290	Tc^r	29
pCVD626	pRK290::(*ace zot ctxAB*) Tc^r	This study

combinant DNA techniques described by Sambrook et al. (30) were employed. DNA fill-in reactions were performed with the polymerase, deoxynucleoside triphosphates, and buffers supplied in the Sequenase version 1.0 enzyme kit (US Biochemical Corp., Cleveland, Ohio); all other restriction endonucleases, enzymes, and oligonucleotide linkers were purchased from Life Technologies or New England Biolabs, Beverly, Mass. Bacterial mating procedures were performed as described previously (13).

DNA hybridizations. DNA hybridization experiments were performed with the following radiolabeled DNA probes: the 1.1-kb fragment generated by the polymerase chain reaction from the *Xba*I restriction site in *ctxA* to the 3′ end of *ctxB* (probe 2 in Fig. 1); the 3.9-kb *Ava*I-*Hin*cII fragment isolated from pCVD626, containing nearly the entire core region (probe 1 in Fig. 1); and the intact plasmid pCVD43, which carries the El Tor hemolysin gene (*hlyA*). Fragment or plasmid probes were radiolabeled with α-^{32}P by primer extension.

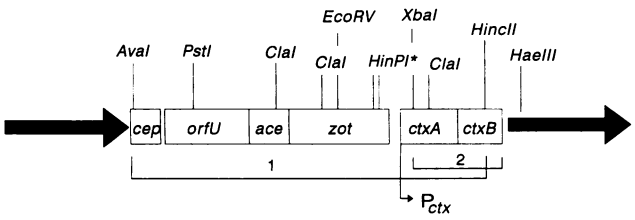

FIG. 1. Diagram of restriction sites and toxin genes constituting the *V. cholerae* El Tor E7946 virulence cassette. Open reading frames are indicated as boxes, and the thick arrows flanking the virulence cassette represent the RS1 elements. Internal core region *cep-orfU-ace-zot-ctx* and *ctxB* gene fragment probes are indicated by brackets 1 and 2, respectively. P_{ctx}, CT gene promoter; *, two adjacent *Hin*PI sites.

Phenotypic assays. Expression of CT was quantitated by a modified GM_1-enzyme-linked immunosorbent assay (ELISA) (28). Briefly, strains were grown overnight in AKI medium at 30°C with shaking and then centrifuged at 3,200 × *g* for 15 min, and the supernatant was assayed. Microtiter plate wells were coated with 100 μl of GM_1 ganglioside in carbonate buffer (pH 9.6) and then blocked with 5% fetal bovine serum in phosphate-buffered saline (PBS). All incubations were at 37°C, and plates were washed three to four times between all steps with PBS–0.5% Tween 20. Culture supernatants were added (50 μl) to the top wells, serially diluted 1:2 down the plate, and the wells were incubated for 2 h. CT (List Biologicals; 200 ng/ml), diluted 1:5,000, was used to generate a standard curve. Plates were incubated for 1 h with rabbit anti-CT antiserum followed by a 1-h incubation with goat anti-rabbit immunoglobulin G–alkaline phosphatase conjugate (Sigma) diluted 1:500 in PBS. Plates were developed with a Kirkegaard & Perry Laboratories substrate kit and read on a Titertek multiscan MCC plate reader at a wavelength of 405 nm.

Hemolytic activity was tested on Trypticase soy agar with 5% sheep erythrocytes (BBL, Cockeysville, Md.).

Electroporation. Electroporation was performed as described previously (23). Briefly, overnight *E. coli* cultures were diluted 1:20 in 100 ml of L broth and grown to an optical density at 590 nm of 0.6. Cells were harvested by centrifugation, washed twice, and resuspended in 5 ml of buffer H (137 mM sucrose, 1 mM HEPES [*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid] [pH 8], 10% glycerol). Approximately 200 ng of plasmid DNA was added to 200 μl of competent cells. Conditions for electroporation were 2.4 kV, 200 Ω, and 25 μF. Immediately after electroporation,

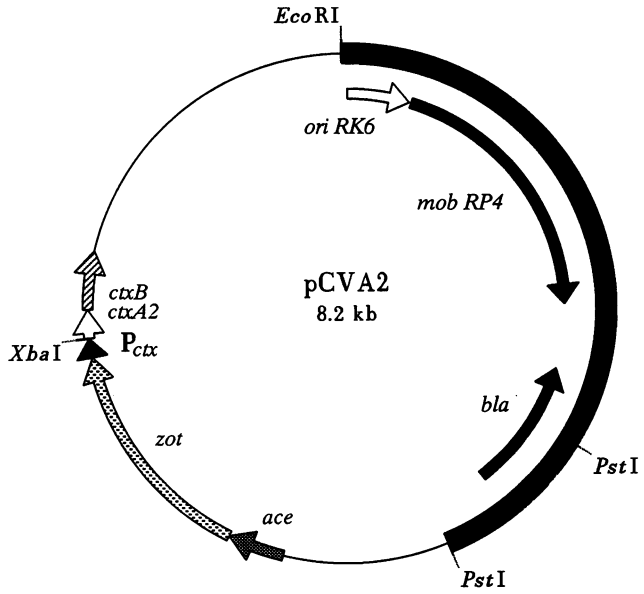

FIG. 2. Map of suicide plasmid pCVA2. Thin lines represent *V. cholerae* sequences cloned from pBB168, and solid boxed regions represent vector pGP704 DNA. Gene position and direction of transcription are indicated by arrows. The *ctx* promoter (P_{ctx}) is indicated as a solid arrowhead. See text for details.

1 ml of SOC medium was added and the bacteria were incubated at 37°C for 1 h and plated onto L agar with ampicillin or $HgCl_2$.

Ussing chambers. To test for enterotoxic activity, *V. cholerae* culture supernatants were tested on rabbit ileal tissue mounted in Ussing chambers. Culture supernatants were prepared and tested as described previously (3, 7). Culture supernatants were size fractionated into fractions <10 kDa and >10 kDa to control for the nonspecific background effects occasionally observed as a result of small molecules present in the culture medium.

Construction of pCVD626. A cosmid library of *V. cholerae* E7946 constructed in pHC79 was screened by colony blot hybridization with a CT B subunit gene probe. One *ctxB*$^+$ recombinant was mapped by restriction enzyme digestion and was found to carry the entire core region. This clone was designated pCVD623. Plasmid pCVD623 was digested with *Bgl*II, and a 7.0-kb fragment was isolated and ligated into pRK290. The ligation mixture was transformed into *E. coli* DH5α, and plasmids from tetracycline-resistant transformants were analyzed by restriction enzyme digestion. One plasmid carrying the 7.0-kb *Bgl*II insert was designated pCVD626.

Construction of pCVA2. Plasmid pCVA2 consists of a 4.7-kb fragment containing the *ctxA2*, *ace*, *zot*, and *ctxB* genes cloned into pGP704 (Fig. 2). Because pGP704 has multiple *Pst*I sites, it was partially digested with *Pst*I to generate linear molecules, which were subsequently isolated by agarose gel electrophoresis, purified, and then digested with *Eco*RI. Plasmid pBB168 (3), containing a 4.7-kb *Pst*I-*Eco*RI fragment carrying *ctxA2*, *ace*, *zot*, and *ctxB*, was digested to completion with *Pst*I-*Eco*RI, and the 4.7-kb fragment was ligated into pGP704 cleaved with *Pst*I-*Eco*RI. The ligation mixture was transformed into *E. coli* SY327λ*pir*, and plasmids from the ampicillin-resistant (Ap^r) transformants were analyzed by restriction enzyme digestion. One plasmid carrying the 4.7-kb *ace-ctxB* insert was designated pCVA2.

Construction of pCVD621 and pCVD622.2B. A 1.4-kb *Hae*III-*Hin*PI fragment containing *ctxB* and P_{ctx} but lacking *ctxA1* was isolated from pCVD30 (13) (Fig. 3). This fragment was filled in and blunt end ligated to phosphorylated *Kpn*I linkers. Following digestion with *Kpn*I, the fragment was ligated into pCVD315 digested with *Kpn*I and transformed into *E. coli* DH5α. The transformants were screened for inserts, and a clone containing the desired insert was designated pCVD621. pCVD621 was digested with *Kpn*I, and the 1.4-kb fragment was ligated into pJMK12, which was linearized with *Kpn*I and transformed into *E. coli* SY327λ*pir*. pJMK12 contained the *V. cholerae hlyA* gene, into which mercury resistance (*mer*) genes have been cloned. Transformants were screened by restriction endonuclease analysis and by resistance to ampicillin and $HgCl_2$. One of 22 recombinant clones screened had a single insert in the desired *Kpn*I site and was designated pCVD622.2B.

Selection of chromosomal recombinants. *V. cholerae* transconjugants containing chromosomal insertion mutations were selected on L agar with ampicillin and polymyxin B; colonies were restreaked for purity on L agar with ampicillin. To select for a second recombination event, in which the suicide vector (Ap^r) was deleted from the chromosome, a single colony was subcultured into L broth and grown to an optical density at 590 nm of 1.0. The culture was then plated onto L agar, and the resulting colonies were replica plated onto L agar with ampicillin or $HgCl_2$.

RESULTS AND DISCUSSION

Pathogenic *V. cholerae* strains contain a highly conserved 4.5-kb core region (25), which contains three toxin genes: *ace*, *zot*, and *ctxAB* (3, 34). In many strains of the El Tor biotype, this 4.5-kb region is tandemly duplicated and flanked by one or more copies of a 2.7-kb element called RS1 (25). Since deletion of the *ctx* genes alone in first-generation recombinant vaccine candidates from *V. cholerae* 395 and N16961 (21) did not completely eliminate the potential of these strains to cause diarrhea or other adverse reactions, we removed the entire 4.5-kb region containing *ctx*, *zot*, and *ace*. Construction of CVD110 involved two successive rounds of mutagenesis using the suicide vector pGP704; use of pGP704 allowed us to mutate two regions of the *V. cholerae* chromosome with a minimum number of strain passages, thereby reducing the chance of any spontaneous mutations altering the strain.

Construction of *V. cholerae* CVD109. To construct *V. cholerae* CVD109 lacking all three toxin genes, we selected for derivatives in which recombination between RS1 elements resulted in deletion of all 4.5-kb core regions. A 4.7-kb fragment containing *ace*, *zot*, and *ctxB* was subcloned into the suicide vector pGP704 as described above (Fig. 2). The resulting plasmid, pCVA2, was transformed into *E. coli* SM10λ*pir* and mobilized into *V. cholerae* E7946 by conjugation. Because *V. cholerae* does not provide the necessary π protein required for plasmid replication, pCVA2 is not stably maintained extrachromosomally. A single crossover event between the homologous sequences on the plasmid and the *V. cholerae* core region results in an Ap^r cointegrate in which the entire plasmid has integrated into the host genome. Ap^r transconjugants were grown in L broth without selective pressure and plated onto L agar to allow a second recombination event, in which a crossover between RS1 elements flanking the toxin genes can lead to loss of the toxin

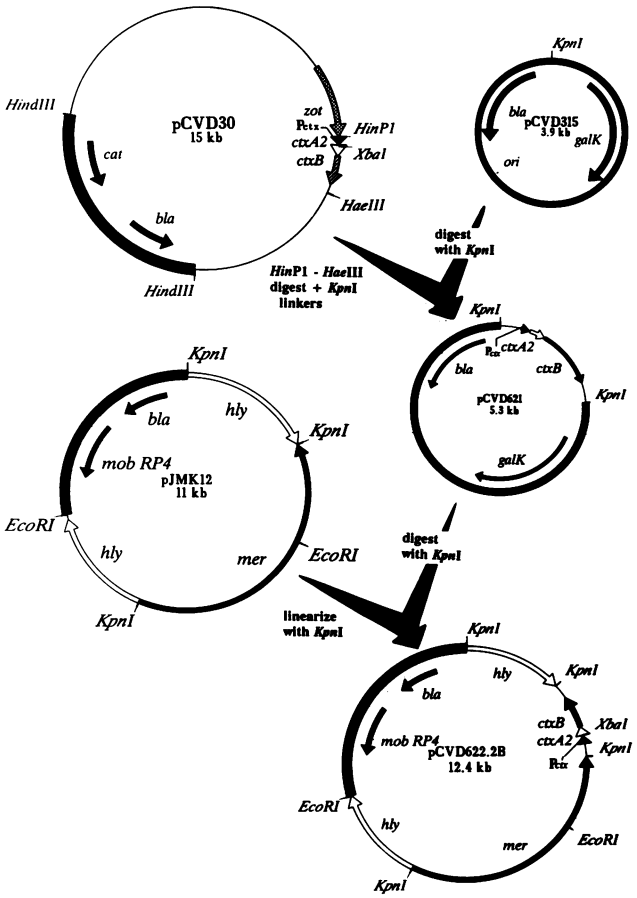

FIG. 3. Construction of plasmids pCVD621 and pCVD622.2B. Solid boxed regions represent vector DNA. Gene position and direction of transcription are indicated by arrows. The *ctx* promoter (P_{ctx}) is indicated as a solid arrowhead. See text for details.

genes. The insertion mutation was highly unstable if the strain was not maintained under selective pressure, and after being replica plated onto ampicillin agar, ca. 50% of the colonies were ampicillin sensitive (Ap^s), indicating that the insertion had been lost. Ap^s colonies were screened by DNA hybridization using a 4.1-kb toxin gene probe (Fig. 1). Southern blot analysis of chromosomal DNA isolated from one colony confirmed that the core region containing *ace*, *zot*, and *ctx* had been deleted (Fig. 4); this colony was designated CVD109.

Construction of *V. cholerae* CVD110. Because immunity against the B subunit of CT may play a synergistic role with colonization and antibacterial immunity to cholera (22, 31) and because the *hlyA* locus can be mutated without adverse effects on immunogenicity (21), we reintroduced the *ctxB* gene along with the native *ctx* promoter into the *hlyA* locus of the *V. cholerae* chromosome. In addition to the *ctxB* sequences, genes encoding mercury resistance (*mer*) were also inserted into the *hlyA* locus. The insertion of *mer* genes distinguishes CVD110 from wild-type *V. cholerae* O1 strains both phenotypically and genetically. Phenotypically, CVD110 has the ability to grow on L agar containing 50 μM $HgCl_2$, unlike wild-type *V. cholerae* O1. The mercury resistance gene also serves as a genetic marker, and DNA hybridization with a *mer* gene probe will distinguish CVD110 from wild-type *V. cholerae* O1 strains.

Plasmid pJMK12, a derivative of pGP704 consisting of the El Tor hemolysin *hlyA* gene inactivated by insertion of a 4.2-kb mercury resistance gene (15), was utilized for this purpose. Plasmid pCVD622.2B, described above, was electroporated into *E. coli* SM10λ*pir* and subsequently mobilized into *V. cholerae* CVD109 by mating. Selection for Ap^r transconjugants yielded cointegrates in which a single crossover event had recombined pCVD622.2B into the host chromosome. Resolution of the cointegrate, detected by ampicillin sensitivity, yielded one of two possible outcomes (Fig. 5). One outcome was the loss of the entire pCVD622.2B plasmid, yielding a strain identical to CVD109. The other possible outcome was the loss of the pGP704 plasmid sequences but retention of the *ctxB* and *mer* sequences in the *hlyA* locus. To detect the second outcome, *V. cholerae* isolates were tested for mercury resistance on L agar containing 30 μM $HgCl_2$ and for loss of hemolytic activity on Trypticase soy agar with 5% sheep erythrocytes. Insertion within the hemolysin gene of CVD110 was verified by a predicted increase in size of the *Pst*I hemolysin gene fragment compared with that of the parent strain, E7946 (Fig. 6A). Insertion of *ctxB* into the CVD110 chromosome was confirmed by hybridization with a *ctxB* fragment probe (Fig. 6B). The two bands seen in lane a of the autoradiograph shown in Fig. 6B are the result of the tandem duplications of the core region.

Expression of *ctxB* in *V. cholerae* constructs. *V. cholerae* vaccine constructs were assayed by GM_1-ELISA for their ability to produce CT B subunit. Expression levels of *V. cholerae* CVD110 B subunit were approximately 70% that of expression levels of E7946, with averages of 16.38 and 23.25 ng/ml ($P < 0.005$, standard deviation = 4.57, where $n = 10$), respectively. *V. cholerae* CVD109 produced no detectable amounts of B subunit. The slightly reduced level of CVD110 may be the result of a gene dosage effect since E7946 contains multiple amplifiable copies of the *ctx* operon (25) whereas CVD110 contains a single stable copy of *ctxB*. However, we cannot rule out the possibility that expression of *ctxB* may be influenced by the adjacent *hlyA* or *mer* genes. In any event, the level of B subunit expression observed with CVD110 should be sufficient since volunteers immunized with *V. cholerae* JBK70 completely lacking B subunit

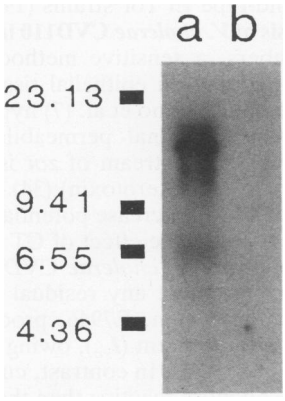

FIG. 4. Southern blot hybridization of chromosomal DNA digested with *Hin*dIII and probed with ^{32}P-labeled core region gene probe 1 (Fig. 1). Lambda *Hin*dIII DNA molecular weight markers (in kilobases) are shown on the left. Lanes: a, E7946; b, CVD109.

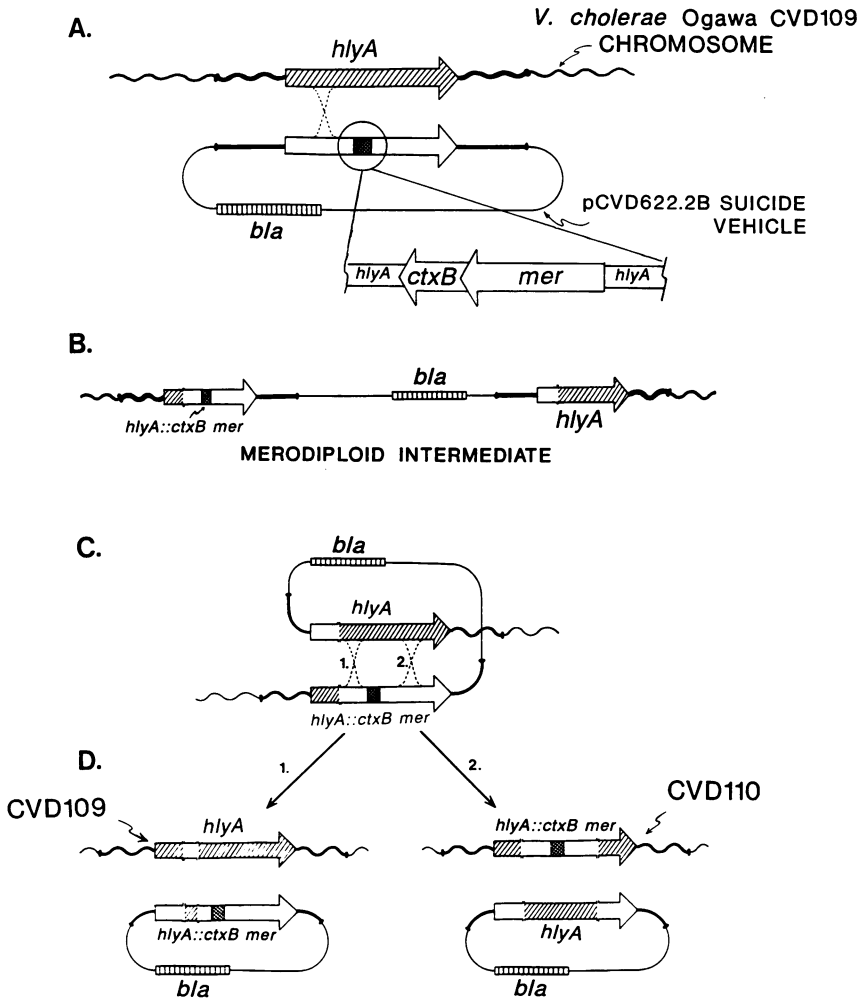

FIG. 5. Model for the formation and resolution of *V. cholerae* chromosomal cointegrates by homologous recombination. (A) Integration of suicide plasmid pCVD622.2B, containing the mercury resistance gene (*mer*) and the CT B subunit (*ctxB*) flanked by homologous hemolysin (*hly*) sequences, into the *V. cholerae hly* locus by a single crossover event. (B) Chromosomal cointegrate which contains one mutated (left) and one wild-type (right) copy of the *hly* locus. (C) Excision of the pCVD622.2B suicide vector from the chromosome by a second crossover event, which can occur either upstream (arrow 1) or downstream (arrow 2) of the inserted sequence. (D) Resolved constructs: arrow 1, wild-type *hly* locus; arrow 2, *ctxB* and *mer* introduced into the *hly* locus.

were protected from disease as well as volunteers previously infected with wild-type El Tor strains (19, 21).

In vitro analysis of *V. cholerae* CVD110 in Ussing chambers. In Ussing chambers, a sensitive method for studying the effects of toxin activity on epithelial tissue, Zot decreases tissue resistance, and Fasano et al. (7) hypothesized that the resulting increased intestinal permeability could lead to diarrhea. Immediately upstream of *zot* is a gene encoding Ace (accessory cholera enterotoxin) (34). The effect of Ace in Ussing chambers is to increase potential difference across the tissue, which is also the effect of CT in this system (7). Culture supernatants of *V. cholerae* CVD110 were tested in Ussing chambers to detect any residual toxin activity. As expected, the parent strain, E7946, produces a strong increase in short-circuit current (I_{sc}), owing to the presence of the *ctx*, *zot*, and *ace* genes. In contrast, culture supernatants of CVD110 were no more reactive than the negative control, PBS (Fig. 7).

Another toxin of *V. cholerae* which has been reported to cause enterotoxic activity is the cytotoxin/hemolysin (24). We previously constructed attenuated *V. cholerae* strains, CVD104 and CVD105, which contain a deletion in the *hlyA* gene encoding this toxin in addition to the deletion in the *ctxA* gene (14). When fed to volunteers, these strains still caused diarrhea (21). While these results do not totally rule out a role for the cytotoxin/hemolysin in the diarrhea observed with the *ctxA* mutants, they at least show that this toxin is not the only agent responsible for this diarrhea. The *hlyA* gene of CVD110 is inactivated by deletion of a 400-bp fragment and insertion of the *mer* genes into the site. When tested in Ussing chambers, CVD110 gave significantly reduced activity compared with the parent strain, E7946 (Fig. 7). In fact, the activity seen with CVD110 was not significantly different from that seen with the PBS control, an outcome which strongly suggests that all enterotoxic activity has been eliminated. Alm et al. (1) have suggested that enterotoxic activity remains in the N-terminal portion of the cytotoxin/hemolysin; most of the implicated N-terminal region is retained in the CVD104 and CVD105 constructs. However, interpretation of the enterotoxin studies of Alm et

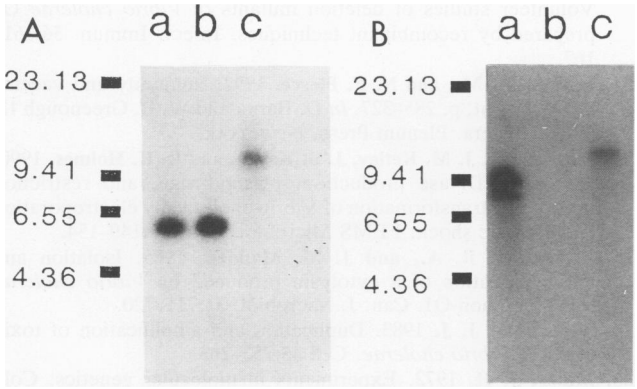

FIG. 6. Southern blot of *V. cholerae* chromosomal DNA digested with *Pst*I and hybridized with either a ^{32}P-labeled *hly* plasmid probe (A) or a ^{32}P-labeled *ctxB* fragment probe (B). Lambda *Hin*dIII DNA molecular weight markers are shown on the left. Lanes: a, E7946; b, CVD109; and c, CVD110.

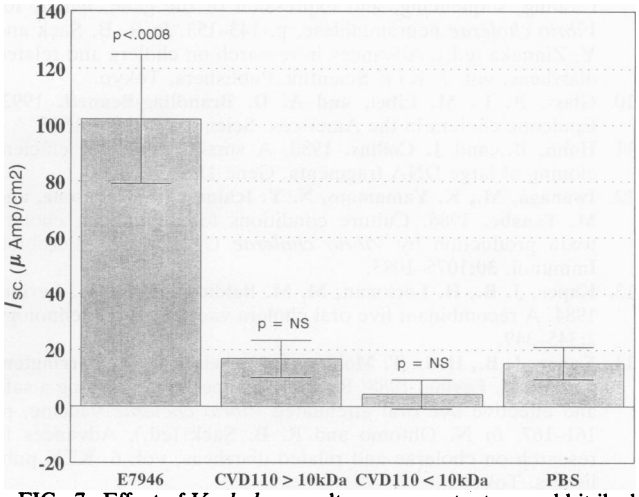

FIG. 7. Effect of *V. cholerae* culture supernatants on rabbit ileal tissue, measured in Ussing chambers. Values are means for four to eight animals; error bars show standard error of the mean. *P* values are given for each culture supernatant compared with the negative control, PBS. Culture supernatants of CVD110 were divided into <10-kDa and >10-kDa fractions. NS, not significant; Isc, short-circuit current.

al. is complicated by the presence of one or more of the CT, Zot, and Ace toxins in the strains examined. Since CVD110 containing the same N-terminal region of the cytotoxin/hemolysin present in CVD104 and CVD105 gave no enterotoxic activity in Ussing chambers, we believe that any activity expressed from this region would not lead to reactogenicity. In addition to the in vitro assay mentioned above, CVD110 has also been tested for reactogenicity in an animal model. Using ligated rabbit ileal loops, Trucksis et al. (34) demonstrated that CVD110 was negative for fluid accumulation (less than 0.5 ml/cm).

For more than a century, the goal of an effective vaccine against cholera has been pursued. One highly promising candidate vaccine is *V. cholerae* CVD103-HgR, a derivative of classical strain 569B. In volunteer studies, vaccination with a single dose of CVD103-HgR confers complete protection against severe, cholera-like illness after experimental challenge with virulent classical or El Tor *V. cholerae* strains (20, 32). However, some volunteers vaccinated with CVD103-HgR, although completely protected from severe diarrhea, experienced mild diarrhea after experimental challenge with wild-type strains of the El Tor biotype (20). Since the predominant *V. cholerae* biotype in the world today is El Tor, an attenuated El Tor vaccine strain may be important for maximum protection against disease. However, volunteer studies with wild-type strains have shown that an initial infection with classical *V. cholerae* confers 100% protection against homologous rechallenge, whereas an initial infection with El Tor confers ca. 90% protection against homologous rechallenge (16). Recent studies in Bangladesh have also suggested that infection with a classical strain is a more powerful immunizing experience than infection with an El Tor strain (6). Thus, a bivalent cholera vaccine consisting of CVD103-HgR and an attenuated El Tor strain such as CVD110 may provide optimal protection.

ACKNOWLEDGMENTS

This work was supported by Public Health Service grant number AI 19716 from the National Institutes of Health.

We thank Mardi Reymann for her helpful advice with the CT assays and Myron Levine and Michelle Trucksis for their critical review of the manuscript.

ADDENDUM IN PROOF

The attenuated *V. cholerae* O1 vaccine strain described in this article, CVD 110, was recently evaluated in volunteers (C. O. Tacket, G. Losonsky, J. P. Nataro, S. J. Cryz, R. Edelman, A. Fasano, J. Michalski, J. B. Kaper, and M. M. Levine, J. Infect. Dis., in press). The strain engendered high antibody titers but still caused diarrhea and other symptoms, thus indicating that there are additional factors responsible for the adverse reactions of Δ*ctx V. cholerae* strains.

REFERENCES

1. **Alm, R. A., G. Mayrhofer, I. Kotlarski, and P. Manning.** 1991. Amino-terminal domain of the El Tor haemolysin of *Vibrio cholerae* O1 is expressed in classical strains and is cytotoxic. Vaccine **9**:588–594.
2. **Barrineau, P., P. Gilbert, W. J. Jackson, C. S. Jones, A. O. Summers, and S. Wisdom.** 1984. The DNA sequence of the mercury resistance operon of the IncFII plasmid NR1. J. Mol. Appl. Genet. **2**:601–619.
3. **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.
4. **Bethesda Research Laboratories.** 1986. BRL pUC host: *E. coli* DH5 alpha competent cells. Focus **8**:9.
5. **Cash, R. A., S. I. Music, J. P. Libonati, J. P. Craig, N. F. Pierce, and R. B. Hornick.** 1974. Response of man to infection with *Vibrio cholerae*. II. Protection from illness afforded by previous disease and vaccine. J. Infect. Dis. **130**:325–333.
6. **Clemens, J. D., F. van Loon, D. A. Sack, M. R. Rao, F. Ahmed, J. Chakraborty, B. A. Kay, M. R. Khan, M. Yunus, J. R. Harris, A.-M. Svennerholm, and J. Holmgren.** 1991. Biotype as determinant of natural immunising effect of cholera. Lancet **337**:883–884.
7. **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.
8. **Feachem, R. G.** 1981. Environmental aspects of cholera epidemiology. I. A review of selected reports of endemic and epidemic situations during 1961–1980. Trop. Dis. Bull. **78**:675–698.
9. **Galen, J. E., E. R. Vimr, L. Lawrisuk, and J. B. Kaper.** 1990.

Cloning, sequencing, and expression of the gene, *nanH*, for *Vibrio cholerae* neuraminidase, p. 143–153. *In* R. B. Sack and Y. Zinnaka (ed.), Advances in research on cholera and related diarrheas, vol. 7. KTK Scientific Publishers, Tokyo.

10. **Glass, R. I., M. Libel, and A. D. Brandling-Bennett.** 1992. Epidemic cholera in the Americas. Science **256:**1524–1525.
11. **Hohn, B., and J. Collins.** 1980. A small cosmid for efficient cloning of large DNA fragments. Gene **11:**291–298.
12. **Iwanaga, M., K. Yamamoto, N. 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. **Kaper, J. B., H. Lockman, M. M. Baldini, and M. M. Levine.** 1984. A recombinant live oral cholera vaccine. Bio/Technology **2:**345–349.
14. **Kaper, J. B., H. L. T. Mobley, J. Michalski, D. A. Herrington, and M. M. Levine.** 1988. Recent advances in developing a safe and effective live oral attenuated *Vibrio cholerae* vaccine, p. 161–167. *In* N. Ohtomo and R. B. Sack (ed.), Advances in research on cholerae and related diarrheas, vol. 6. KTK publishers, Tokyo.
15. **Ketley, J. M., J. Michalski, J. Galen, M. M. Levine, and J. B. Kaper.** 1993. Construction of genetically marked *Vibrio cholerae* O1 vaccine strains. FEMS Microbiol. Lett. **111:**15–22.
16. **Levine, M. M.** 1980. Immunity to cholera as evaluated in volunteers, p. 195–203. *In* O. Ouchterlony and J. Holmgren (ed.), Cholera and related diarrheas. Proceedings of the 43rd Nobel symposium, Stockholm, 1978. S. Karger, Basel.
17. **Levine, M. M., R. E. Black, M. L. Clements, L. Cisneros, D. R. Nalin, and C. R. Young.** 1981. Duration of infection-derived immunity to cholera. J. Infect. Dis. **143:**818–820.
18. **Levine, M. M., R. E. Black, M. L. Clements, D. R. Nalin, L. Cisneros, and R. A. Finkelstein.** 1981. Volunteer studies in development of vaccines against cholera and enterotoxigenic *Escherichia coli*: a review, p. 443–459. *In* T. Holme, J. Holmgren, M. H. Merson, and R. Mollby (ed.), Acute enteric infections in children. New prospects for treatment and prevention. Elsevier, Amsterdam.
19. **Levine, M. M., J. B. Kaper, R. E. Black, and M. L. Clements.** 1983. New knowledge of bacterial enteric infections as applied to vaccine development. Microbiol. Rev. **47:**510–550.
20. **Levine, M. M., J. B. Kaper, D. Herrington, J. Ketley, G. Losonsky, C. O. Tacket, B. Tall, and S. Cryz.** 1988. Safety, immunogenicity, and efficacy of recombinant live oral cholera vaccines, CVD 103 and CVD 103-HgR. Lancet **ii:**467–470.
21. **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.
22. **Levine, M. M., and N. F. Pierce.** 1992. Immunity and vaccine development, p. 285–327. *In* D. Barua and W. B. Greenough III (ed.), Cholera. Plenum Press, New York.
23. **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. **56:**149–154.
24. **McCardell, B. A., and J. M. Madden.** 1985. Isolation and characterization of a cytolysin produced by *Vibrio cholerae* serogroup non-O1. Can. J. Microbiol. **31:**711–720.
25. **Mekalanos, J. J.** 1983. Duplication and amplification of toxin genes in *Vibrio cholerae*. Cell **35:**253–263.
26. **Miller, J. H.** 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
27. **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.
28. **Ristano, P. A., M. M. Levine, and C. R. Young.** 1983. Improved GM1-enzyme-linked immunosorbent assay for detection of *Escherichia coli* heat-labile enterotoxin. J. Clin. Microbiol. **18:**808–815.
29. **Ruvkun, G. R., and F. M. Ausubel.** 1981. A general method for site-directed mutagenesis in prokaryotes. Nature (London) **289:**85–88.
30. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
31. **Svennerholm, A. M., and J. Holmgren.** 1976. Synergistic protective effect in rabbits of immunization with *Vibrio cholerae* lipopolysaccharide and toxin/toxoid. Infect. Immun. **13:**735–740.
32. **Tacket, C. O., G. Losonsky, J. P. Nataro, S. J. Cryz, R. Edelman, J. B. Kaper, and M. M. Levine.** 1992. Onset and duration of protective immunity in challenged volunteers after vaccination with live oral cholera vaccine CVD 103-HgR. J. Infect. Dis. **166:**837–841.
33. **Tauxe, R. V., and P. A. Blake.** 1992. Epidemic cholera in Latin America. JAMA **267:**1388–1390.
34. **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.