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Accessory cholera enterotoxin (Ace), the third toxin of a *Vibrio cholerae* virulence cassette

(cystic fibrosis/bacterial pathogenesis/diarrhea)

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ABSTRACT **_Vibrio cholerae_ causes the potentially lethal disease cholera through the elaboration of the intestinal secretogen cholera toxin. A second toxin of _V. cholerae_, Zot, decreases intestinal tissue resistance by modifying intercellular tight junctions. In this report, a third toxin of _V. cholerae_, Ace (_a_ccessory _c_holera _e_nterotoxin), is described. Ace increases short-circuit current in Ussing chambers and causes fluid secretion in ligated rabbit ileal loops. The predicted protein sequence of Ace shows striking similarity to eukaryotic ion-transporting ATPases, including the product of the cystic fibrosis gene. The gene encoding Ace is located immediately upstream of the genes encoding Zot and cholera toxin. The _ctx_, _zot_, and _ace_ genes, which are located on a dynamic sector of the chromosome, comprise a _V. cholerae_ "virulence cassette."**

Development of live attenuated *Vibrio cholerae* oral vaccines by recombinant technology initially targeted the genes encoding cholera toxin (*ctx*) for deletion. Although these initial vaccine candidates, such as JBK70 and CVD101, elicited high antibody responses and did not cause severe diarrhea in volunteers, more than half of the vaccinees developed mild to moderate diarrhea and many experienced abdominal cramps, anorexia, and low-grade fever (1). In search of an additional toxin which could explain this reactogenicity, we reported a second toxin, Zot (*z*onula *o*ccludens *t*oxin), which decreases ileal tissue resistance by affecting intercellular tight junctions (2). The diarrhea observed in volunteers fed Δ*ctx V. cholerae* vaccine strains was hypothesized to be caused by alteration of tight junctions, with a subsequent increase in intestinal permeability.

The gene encoding Zot is located immediately upstream of *ctx* (3) on a 4.5-kb region termed the "core region" (4). In many strains of *V. cholerae*, this 4.5-kb region is flanked by one or more copies of a 2.7-kb sequence called RS1; homologous recombination between RS1 elements can lead to tandem amplification of the 4.5-kb region (4). Since the *zot* and *ctx* genes comprise only 55% of the 4.5-kb core region, we investigated the potential pathogenic role of the remaining portion of this region. Our results show that the gene for a third toxin is included in this region‖ and that this third toxin increases the short-circuit current (I_{sc}) in Ussing chambers and causes fluid secretion in ligated rabbit ileal loops.

MATERIALS AND METHODS

Bacterial Strains. *V. cholerae* E7946 is an El Tor Ogawa strain previously demonstrated to produce cholera in volunteers (5). Strain CVD110 was constructed from E7946 by homologous recombination of the RS1 elements, resulting in deletion of the core region (J.M., J.E.G., A.F., and J.B.K., unpublished work). In brief, cloned *ctx* and *zot* sequences were inserted into the suicide vector pGP704 (7) and conjugated into E7946. Homologous recombination of the *ctx*, *zot*, and vector sequences into the chromosome yielded ampicillin-resistant colonies. A second recombination event between the flanking RS1 elements resulted in deletion of *ace*, *ctx*, *zot*, and vector sequences that was detected by loss of ampicillin resistance. *Escherichia coli* DH5α was used as a host for cloning experiments.

Construction of Plasmids. DNA manipulations were carried out as described (8); plasmid constructs were transformed into *V. cholerae* CVD110 by electroporation (9). To construct plasmid pCVD630, a cosmid library was constructed with *V. cholerae* E7946 chromosomal DNA, and a recombinant clone containing *ctx* and *zot* was isolated. A 2.9-kb *Eco*RV fragment immediately upstream of *ctx* and containing the first 391 bp of *zot* was subcloned into plasmid vector pCVD315 (10), generating pCVD630. Plasmid pCVD630A was created by deletion of a 309-bp *Bsu*36I–*Sty* I fragment that resulted in an in-frame mutation in open reading frame *orfU*. Plasmid pCVD371 contains the cloned *ace* gene generated by PCR using Deep Vent DNA polymerase (New England Biolabs). Two primers were employed: a 26-base synthetic primer, 5′-TAAGGATGTGCTTATGATGGACACCC-3′, which is complementary to the 5′ end of *ace* and contains a ribosome binding site 5′ to the start codon, and a 25-base synthetic primer, 5′-CGTGATGAATAAAGATACTCATAGG-3′, which is complementary to the 3′ end of *ace*. The resulting product, containing *ace*, was ligated into the filled-in *Bam*HI site of pTTQ181K to produce pCVD371. [Plasmid pTTQ181K was constructed by insertion of a *Bam*HI fragment containing the *kan* gene of Tn*5* into the *bla* gene of pTTQ181 (Amersham).]

Plasmid pCVD372 consists of a truncated *orfU* fragment, identical to the intact 5′ end of *orfU* in pCVD630A, generated by PCR and ligated into the filled-in *Bam*HI site of pTTQ181K. Two primers were employed to generate the truncated *orfU* fragment: a 30-base synthetic primer, 5′-TAAGGATGTTTATGCGCTATTTTCTACTG-3′, which is complementary to the 5′ end of *orfU* and contains a ribosome binding site 5′ to the start codon, and a 19-base synthetic primer, 5′-GTGGTAAGGGAACGCACTA-3′, which introduces a stop codon 339 bp into the gene.

Nucleotide Sequence. The nucleotide sequence was determined by using the M13 dideoxy method. Sequence analysis used the programs developed by the Genetics Computer Group at the University of Wisconsin (11).

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Abbreviations: I_{sc}, short-circuit current; PD, potential difference.

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‖The sequence reported in this paper has been deposited in the GenBank data base (accession no. Z22569).

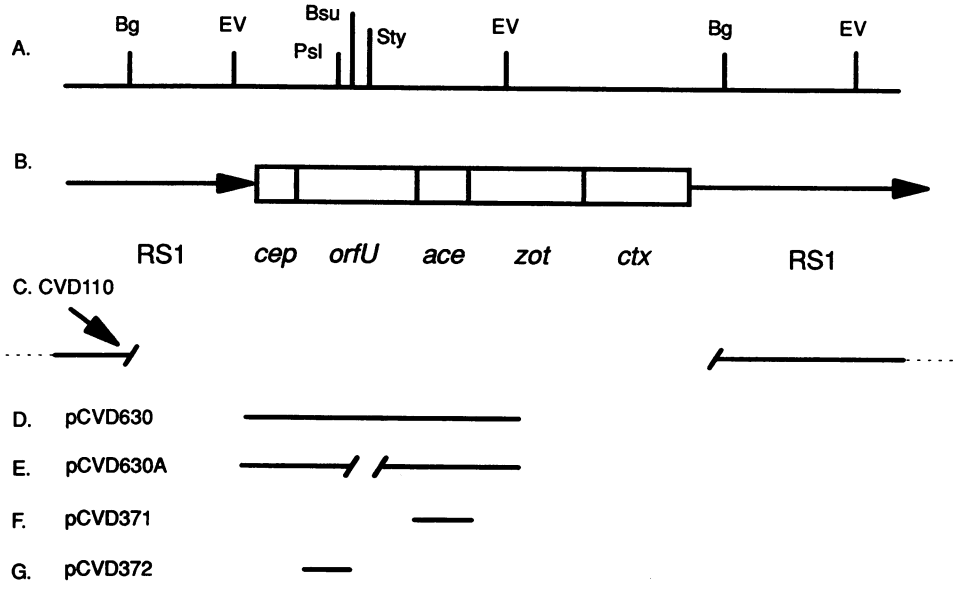

FIG. 1. Bacterial strains and plasmids used in this study. (*A*) Restriction map of the "core region" of *V. cholerae* E7946. Bg, *Bgl* II; EV, *Eco*RV; PsI, *Pst*I; Bsu, *Bsu*36I; Sty, *Sty* I. (*B*) Simplified schematic depicting the core region as a single copy with flanking RS1 elements. Open reading frames identified by DNA sequence analysis in the core region: *cep*, core-encoded pilin; *orfU*, open reading frame of unknown function; *ace*, accessory cholera enterotoxin; *zot*, zonula occludens toxin; *ctx*, cholera toxin operon including *ctxA* and *ctxB*. (*C*) Map of *V. cholerae* strain CVD110, showing the sequences deleted from E7946. (*D*) Plasmid pCVD630, containing the 2.9-kb *Eco*RV fragment immediately upstream of *ctx*. (*E*) pCVD630A, which was constructed from pCVD630 by deletion of a 309-bp *Bsu*36I–*Sty* I fragment, yielding *ace* Δ*orfU*. (*F*) pCVD371, containing the *ace* gene generated by PCR. (*G*) pCVD372, containing the 5′ end of *orfU* generated by PCR.

Ussing-Chamber Experiments. Experiments were performed as described (2) with ileal tissue from adult male New Zealand White rabbits. Rabbits were anesthetized by methoxyflurane inhalation and then euthanized by cardiac puncture with air embolism. Ileal tissue was mounted into Lucite Ussing chambers (1.12-cm^2 aperture) and bathed with Ringer's buffer. Potential difference (PD) was measured, and I_{sc} and tissue conductance (G_t) were calculated (12). Once the tissue reached equilibrium, 300 μl (a 1:33 dilution) of culture supernatant was added to the mucosal side (time 0); 300 μl of the same sample was added to the serosal side to preserve the osmotic balance. At the end of each experiment, 200 μl of 0.5 M glucose (final concentration, 10 mM) was added to the mucosal side of each chamber; only those tissues which showed increased I_{sc} in response to glucose (indicating tissue viability) were included in the analysis.

Overnight cultures of *V. cholerae* CVD110(pCVD630 or pCVD630A) were diluted in L broth containing ampicillin (100 μg/ml) and grown at 37°C with shaking. Bacterial strains CVD110(pTTQ181K), CVD110(pCVD371), and CVD110(pCVD372) were similarly grown in L broth containing kanamycin (50 μg/ml), and isopropyl β-D-thiogalactopyranoside (2 mM) was added to the cultures when they reached OD_{600} of 0.5. Incubation was continued for all cultures for an additional 2 hr. Culture supernatants were filtered through a 0.45-μm filter (Millipore), concentrated by vacuum dialysis (M_r 10,000 cutoff), and suspended with phosphate-buffered saline (PBS) to the original volume.

Ligated Rabbit Ileal Loops. Ileal-loop experiments were conducted essentially as described (13). In brief, rabbits were anesthetized with a mixture of ketamine (50 mg/kg), acepromazine (0.5 mg/kg), and rompun (5–8 mg/kg) and the small intestine was tied off in alternating 10- and 2-cm segments. A

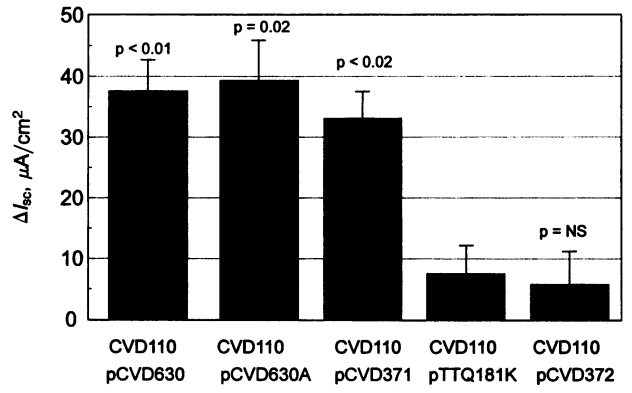

FIG. 2. Effect of *V. cholerae* culture supernatants on ileal I_{sc}. Values are means for three to four animals; error bars show 1 SE (standard error of the mean). The statistical test used was Student's *t* test. *P* values are given for each culture supernatant compared with the negative control *V. cholerae* CVD110(pTTQ181K). NS, not significant.

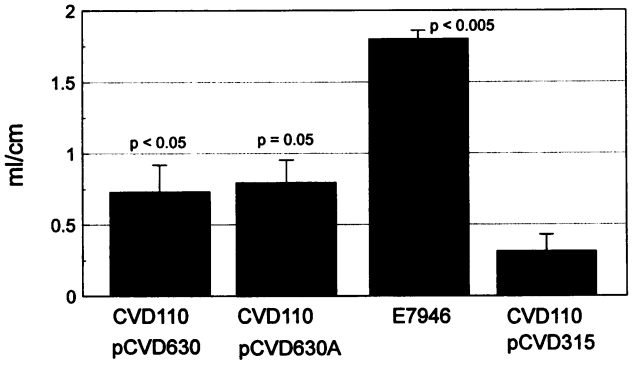

FIG. 3. Effect of *V. cholerae* strains on fluid accumulation in ligated rabbit ileal loops. Values are means; error bars show 1 SE. Each strain was tested in a minimum of three and a maximum of nine different animals. In some animals duplicate loops for strains were performed. The position of each test loop was randomized by an individual not involved in the loop experiments. The surgical and necropsy teams were kept unaware of the position of each strain until after the results were calculated. The statistical test used was Student's *t* test. *P* values are given for each bacterial strain compared with the negative control *V. cholerae* CVD110(pCVD315).

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                    -35              -10                                     M  L  M  M  D  T  L  Y  D  W
1211  TGCACATCATCCGCATGATAGTGCTCGCGATGGCGTACATTCTGGCCGCCATGCTCATTTTGTTTAGGTGATGCTTATGATGGACACCCTTTATGACTGG  1310
       ... I  I  R  M  I  V  L  A  M  A  Y  I  L  A  A  M  L  I  L  F  R  *

      L  I  D  G  F  T  W  L  V  I  K  L  G  I  M  W  I  E  S  K  I  F  V  I  Q  F  F  W  E  M  S  Q  K  V
1311  CTAATTGATGGCTTTACGTGGCTTGTGATCAAGCTCGGTATTATGTGGATTGAGAGCAAGATTTTTGTTATCCAATTCTTCTGGGAGATGTCCCAGAAAG  1410

       I  D  M  F  T  I  Y  P  L  I  Q  Q  A  I  D  M  L  P  P  Q  Y  S  G  F  L  F  F  L  G  L  D  Q  A
1411  TGATTGATATGTTTACCATCTATCCGCTTATCCAACAGGCTATCGATATGCTGCCTCCTCAATACAGCGGCTTTCTGTTCTTTTTAGGGTTAGACCAAGC  1510

      L  A  I  V  L  Q  A  L  M  T  R  F  A  L  R  A  L  N  L  *
1511  GCTGGCTATCGTGCTTCAGGCTTTGATGACCCGTTTTGCCCTGCGAGCGTTAAACCTATGAGTATCTTTATTCATCACGGCGCGCCAGGCTCTTATAAAA  1610
                                                                   M  S  I  F  I  H  H  G  A  P  G  S  Y ...
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FIG. 4. Nucleotide sequence and predicted amino acid sequence of *ace*; nucleotides are numbered as in the complete GenBank entry for *ace* and *orfU* (accession no. Z22569). Possible −35 and −10 promoter sequences are indicated and nucleotides complementary to the 3′-hydroxyl end of *E. coli* 16S rRNA, indicating a potential ribosome binding site, are at positions 1274–1282. The 3′ end of *orfU* is shown immediately upstream of *ace* and the 5′ end of *zot* (3) is shown immediately downstream of *ace*.

l-ml inoculum containing 10^9 colony-forming units of *V. cholerae* was injected into the 10-cm segment and the intestine was returned to the abdomen. Eighteen hours later, the rabbits were euthanized with ketamine (50 mg/kg) and acepromazine (1 mg/kg) followed by cardiac puncture with air embolism, the fluid in the 10-cm segments was measured, and the ratio of fluid (milliliters) to length (centimeters) was calculated.

RESULTS

V. cholerae CVD110, a derivative of the virulent *V. cholerae* El Tor Ogawa strain E7946 (5), was constructed with a deletion of the 4.5-kb core region containing *ctx* and *zot* (Fig. 1). When tested in Ussing chambers, used for measuring net ion transport across isolated intestinal mucosa, CVD110 was no more reactive than the buffer control (data not shown). However, when the 2.9-kb *Eco*RV fragment of the core region containing sequence upstream of *zot* but lacking intact *ctx* and *zot* genes was reintroduced into CVD110 on plasmid pCVD630 (Fig. 1), CVD110(pCVD630) showed a significant increase in I_{sc} relative to the negative control, CVD110-(pTTQ181K) (Fig. 2). The increase in I_{sc} was secondary to an increase in PD, with tissue resistance (R_t) remaining stable. Furthermore, in the *in vivo* sealed infant mouse model (14), CVD110(pCVD630) produced 15% greater fluid accumulation than CVD110 alone (data not shown), again suggesting the presence of an unidentified toxin gene on pCVD630.

DNA sequence analysis of pCVD630 revealed two open reading frames (*orfU* and *ace*) immediately upstream of *zot* (Fig. 1). To determine which open reading frame was responsible for the enterotoxic activity, a deletion in the larger open reading frame, *orfU*, was constructed by deletion of a 309-bp *Bsu*36I–*Sty* I fragment. This deletion mutant, pCVD630A, retained full Ussing-chamber activity when reintroduced into CVD110 (Fig. 2). The *in vitro* enterotoxic activity demonstrated in the Ussing chamber was confirmed in an *in vivo* system, the ligated rabbit ileal loop. Both CVD110(pCVD630), the construct containing *orfU* and *ace*, and CVD110-(pCVD630A), the *orfU* deletion mutant, caused significant fluid accumulation in the ligated ileal loops, an outcome consistent with the Ussing-chamber results (Fig. 3).

To confirm that *ace* rather than the residual 5′ end of *orfU* was responsible for this enterotoxic activity, two subclones containing only the individual open reading frames were constructed by the PCR. In plasmid pCVD372, the amplified fragment of *orfU* contained a ribosome binding site 5′ to the start codon and a stop codon 339 bp into the gene, prematurely terminating translation (Fig. 1). This plasmid, containing the same 5′ fragment of *orfU* present in pCVD630A, showed no increase in I_{sc} and was comparable to the negative control CVD110(pTTQ181K). In contrast, CVD110(pCVD371), containing only the intact open reading frame *ace*, showed an increase in I_{sc} of equal magnitude to the positive control CVD110(pCVD630) (Fig. 2), supporting the role of *ace* in secretory action.

We have designated the open reading frame on construct pCVD371 as *ace*, for *a*ccessory *c*holera *e*nterotoxin. This open reading frame is located upstream from *zot* and *ctx* (Fig. 1). The DNA sequence of the *ace* gene (Fig. 4) includes a 289-bp open reading frame counting from the first ATG. Three of the first four codons are ATG, and it is not clear

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CaPM   828  IILTD........DNFTSIVK..AVMW.GRNVYDSISKFLQFQLT.VNVVAV....IV
SpvB   311  VLMFHHFPDELGEADT.LVSRLLLEYDENPIL..T......QLC.AARTLAY..EGD
RPMCA  828  IILTD........DNFTSIVK..AVMW.GRNVYDSISKFLQFQLT.VNVVAV....IV
ACE      1  MLMMDTLYDWLIDGFTWLVIKLGIMWIESKIF..VIQFF.WEMS.QKVIDMF..TIY

ACE      1  MLMMDTLYDWLIDGFTWLVIKLGIMWIESKIF..VIQFF.WEMS.QKVIDMF..TIY
CFTR   242  RMMMKYRDQRAGKISERLVITSEM..IENIQS..VKAYC.WEEAMEKMIENLRQTEL

CaPM   870  AFDGACI.TQDSPLKAVQMLWVNLIMDT.....LASL.......ALATEPPTESLLL
SpvB   356  GYRRAPVNNMMPPPPPPPPPMMGGN.SSRPKSKWAIVEESKQIQALRY.YSAQGYSV
RPMCA  870  AFTGACITTQDSPLKAVQMLWVNLIMDT.....LASL.......ALATEPPTESLLL
ACE     52  PLIQQAI.DMLPPQYSGFLFFLGLD.QA.....LAIV.....LQALMTRFALRALNL

ACE     52  PLIQQAI.DMLPPQYSGFLFFLGLD.QA.....LAIV.....LQALMTRFALRALNL
CFTR   294  KLTRKAA.YVRYFNSSAFFFSGFFV.VF.....LSVL.....PYALIKGIILRKI..
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FIG. 5. Amino acid sequence comparison of the predicted *ace* gene product and predicted sequence of two ion-transporting ATPases, the human plasma membrane Ca^{2+} pump (CaPM) (16) and the Ca^{2+}-transporting ATPase from rat brain (RPMCA) (17); a virulence protein of *Salmonella dublin*, (SpvB) (19); and the cystic fibrosis transmembrane conductance regulator (CFTR) (18). Boxed residues indicate an identical or similar amino acid residue between Ace and the other sequences. Similar residues are those belonging to the groups P/A/G/S/T, H/K/R, Q/N/E/D, I/L/V/M, and F/W/Y (single-letter code). Sequence alignment was performed with the BESTFIT program. Ace was aligned separately with CFTR to maximize sequence similarity.

which of these is the actual initiation codon. For the purpose of discussion, the first of the three predicted methionines will be designated as residue 1, although the location of a potential ribosome binding site (Fig. 4) suggests that the second or third ATG is the actual initiation codon. Starting from the first methionine, the Ace protein would be a 96-residue peptide with a predicted M_r of 11,300. Interestingly, the termination codon of *ace*, TGA, overlaps with the initiation codon of *zot*, which is in a different reading frame. Exactly the same overlap is seen between the termination codon of *ctxA* and the initiation codon of *ctxB* (15). This arrangement suggests translational coupling of the *ace* and *zot* genes. The *ace* open reading frame immediately follows *orfU*, although the exact intergenic distance depends upon which initiation codon of *ace* proves to be correct.

The predicted amino acid sequence of Ace was compared with the Swiss-Prot data base (June 1992) by the program WORDSEARCH. A striking similarity was found between Ace and a family of ion-transporting ATPases including the human plasma membrane calcium pump (CaPM) (56% similarity) (16), the calcium-transporting ATPase from rat brain (RPMCA) (56% similarity) (17), and the product of the cystic fibrosis gene, the cystic fibrosis transmembrane conductance regulator (CFTR) (42% similarity) (18) (Fig. 5). CaPM and RPMCA are involved in the transport of calcium ions across the membrane and CFTR functions as a chloride ion channel (20). In addition to the similarity with eukaryotic proteins, Ace shows 48% sequence similarity with a virulence protein of *Salmonella dublin*, SpvB, inactivation of which abolished virulence in mice (19).

Ace activity is contained in the $M_r > 10,000$ fraction. A time course showing the change in ΔI_{sc} with supernatants of CVD110(pCVD371) (Ace$^+$) compared with CVD110-(pTTQ181K) (negative control) is presented in Fig. 6. Ace is sensitive to treatment with proteinase K (250 μg/ml, 30 min, 56°C) and boiling (100°C, 10 min) but retains full activity after freezing.

DISCUSSION

We have described an enterotoxin, Ace, produced by *V. cholerae*. This toxin increases I_{sc} in rabbit ileal tissue mounted in Ussing chambers, by increasing the PD. Cultures of *V. cholerae* containing the cloned *ace* gene cause fluid secretion in ligated rabbit ileal loops. Although the volume of fluid induced by *V. cholerae* strains containing *ace* alone are not as great as with strains containing *ace*, *zot*, and *ctx* genes, the observed volumes were significantly greater than those seen with the negative control.

The mechanism by which Ace acts is unknown, but the similarity of Ace to a family of ion-transporting ATPases may provide a clue. Ace is much smaller than the proteins of this family and does not contain an obvious nucleotide binding site. The greatest similarity of Ace and the ion-transporting ATPases is in the hydrophobic transmembrane domains of the latter. Ace is predominantly a hydrophobic protein. Analysis of the predicted protein sequence of Ace for its hydrophobic moment according to the method of Eisenberg *et al.* (21) revealed an α-helical region with a μ_H value of 0.63. This region, located at the C-terminal end of Ace, is depicted in Fig. 7 in a helical wheel format. The amphipathic nature of this 20-residue region, in which nearly all of the hydrophobic residues are on one side of the helix, would be consistent with insertion of Ace multimers in the eukaryotic membrane with the hydrophobic surfaces facing the lipid bilayer and the hydrophilic sides facing the interior of a transmembrane pore. Such a structure is found for the 26-residue δ toxin of *Staphylococcus aureus* (22), which has a variety of effects on eukaryotic tissue, including increasing vascular permeability in guinea pig skin and inhibiting water absorption and increasing cAMP concentration in the ileum (23, 24). A stretch of 19 residues in the amphipathic C-terminal region of Ace shows 47% similarity with residues 2–20 of δ toxin (data not shown).

Both Ace and Zot may therefore contribute to the residual reactogenicity of vaccine candidates JBK70 and CVD101. Although both Ace and Zot toxins are physiologically active in Ussing chambers, they act through different mechanisms: Zot decreases tissue resistance (R_t) whereas Ace increases PD across the epithelial membrane. The increased intestinal permeability induced by Zot may be involved in the constitutional symptoms (fever, anorexia, and abdominal cramps) and/or diarrhea, while the increase in PD seen with Ace, as with other

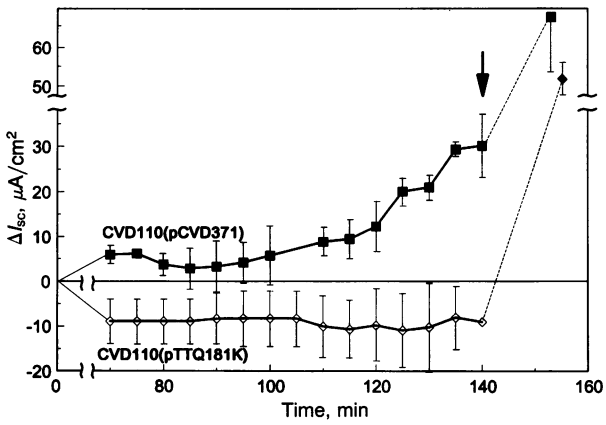

FIG. 6. Time course of ΔI_{sc} of supernatants of *V. cholerae* strains CVD110(pCVD371) (Ace$^+$ construct) (■) versus CVD110(pTTQ181K) (negative control) (◇). Curves represent means ± SD of nine experiments for CVD110(pCVD371) and six experiments for CVD110(pTTQ181K). Time 0 is the time at which the supernatant was added. There is an average lag time of 69 min (range, 55–95 min) before toxin activity is apparent. Arrow indicates time of addition of 10 mM glucose (final concentration) to the mucosal side of each chamber.

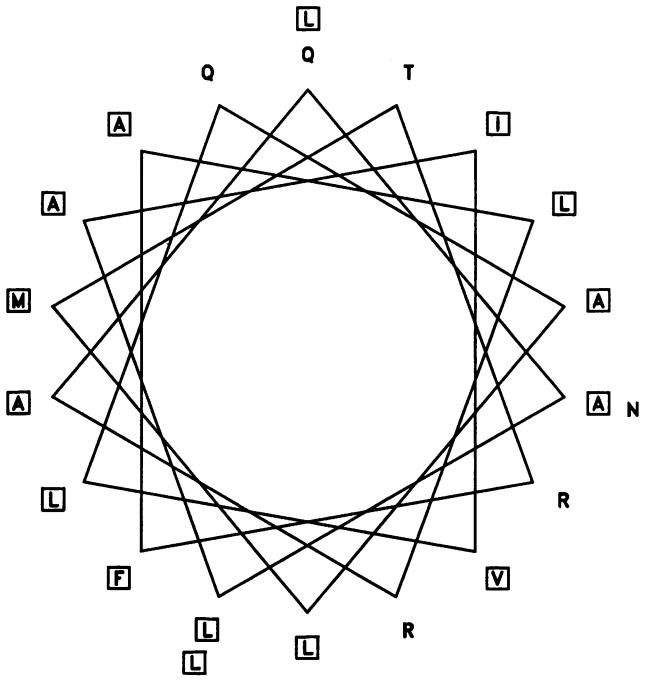

FIG. 7. Potential amphipathic region of Ace. The C-terminal end of the Ace protein containing a predicted α-helix was arranged in the form of a wheel by using the program HELICALWHEEL. Hydrophobic residues are boxed. The glutamine (Q) at the top of the wheel is residue 76 of the predicted amino acid sequence of Ace.

classic enterotoxins, may cause diarrhea. CVD103HgR, a candidate vaccine strain of *V. cholerae* that, in contrast to JBK70 and CVD101, causes no adverse effects in vaccinees (25, 26) contains intact *ace* and *zot* genes, but it is not clear whether mutations in regulatory sequences alter the expression of these genes in this strain. Also, CVD103HgR colonizes the intestine at levels significantly lower than the other vaccine candidates (25). The genetic lesion responsible for this colonization defect may also contribute to the decreased reactogenicity of this vaccine construct.

Naturally occurring nonenterotoxigenic *V. cholerae* cells which lack *ace*, *zot*, and *ctx* sequences (27) do not cause diarrhea in volunteers (28). This preliminary correlation of the presence of the three toxin genes with virulence of *V. cholerae* must be confirmed and extended by specific isogenic mutations before the actual significance of each of these toxins is known. It is intriguing that these three toxin genes are located in tandem on a dynamic region of the *V. cholerae* chromosome and that in many strains of *V. cholerae* these toxin genes are flanked by RS1 elements which have site-specific transposase activity (29). This arrangement can lead to amplification or deletion of all three toxin genes as a unit (4). It has recently been shown that the gene for a colonization factor, *cep* (*c*ore-*e*ncoded *p*ilin), is also located in this region (6). Clustering of virulence genes is emerging as a pattern also seen in other pathogens, such as *Listeria monocytogenes* and *Shigella flexneri*. In *L. monocytogenes*, five virulence genes are located in tandem on the chromosome along with a regulatory gene (30). In *S. flexneri*, a 31-kb DNA segment on the 230-kb virulence plasmid is required for invasion (31). This region contains a cluster of virulence genes and regulatory elements (31), which are highly conserved in pathogenic shigellae and enteroinvasive *E. coli* (32). Thus the clustering of virulence genes in these pathogenic bacteria parallels the organization of toxin genes in *V. cholerae*. The conservation of all three toxins in pathogenic *V. cholerae*, each acting by a different mechanism, suggests the importance of all three in the disease process. The genes encoding these toxins and *cep*, flanked by RS1 elements, may therefore be perceived as a "virulence cassette" of *V. cholerae*.

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