Cloning, Characterization, and Chromosomal Mapping of a Phospholipase (Lecithinase) Produced by *Vibrio cholerae*

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Phospholipases are associated with virulence in bacterial diseases. *Vibrio cholerae* produces a phospholipase (lecithinase), with enzyme production visualized as a zone of clearing around colonies plated on egg yolk agar. The role of phospholipase in gut colonization or disease pathogenesis is unknown. We used the egg yolk agar assay to clone and characterize a gene encoding a phospholipase from *V. cholerae* El Tor strain E7946. Sequence analysis revealed a 1,254-bp open reading frame (*lec*) encoding a 418-amino-acid protein with a predicted molecular weight of 47,600. The predicted sequence exhibits DNA homology to other *Vibrionaceae* phospholipases. A potential signal sequence exists in the predicted amino acid sequence, as does a lipid binding motif found in prokaryotic and eukaryotic phospholipases and lipases. Polyacrylamide gel electrophoresis combined with an egg yolk agarose overlay demonstrated phospholipase activity migrating at a relative molecular weight of 45,000 in preparations of *V. cholerae* and the *Escherichia coli* clone. Restriction mapping and Southern blot analysis revealed that *lec*, *hlyA* (hemolysin), and *hlyC* (lipase) are adjacent on the *V. cholerae* chromosome, and chromosomal digests of several El Tor, classical, and O139 (Bengal) strains demonstrated conservation of this gene arrangement. An in-frame internal deletion of the *lec* gene was constructed and recombined into the chromosome of attenuated *V. cholerae* El Tor strain CVD 110. The resulting mutant lacked lecithinase activity on egg yolk agar but had undiminished reactivity in rabbit ligated ileal loop assays.

The gram-negative bacterium *Vibrio cholerae* is the etiologic agent of cholera, a severe diarrheal illness that causes considerable morbidity and mortality in the developing world. While significant strides toward the development of a live oral attenuated vaccine have been made, progress has been hampered by the reactogenecity of some cholera toxin-negative strains. Live recombinant vaccine candidates derived from serogroups O1 and O139 (Bengal) strains retain the ability to cause mild to moderate diarrhea in volunteer studies (34, 35), despite the deletion of the genes encoding the A subunit of cholera enterotoxin (*ctxA*), zonula occludens toxin (*zot*), and accessory cholera enterotoxin (*ace*) and the inactivation of the hemolysin (*hlyA*) gene (8, 25). In addition, some of the vaccine candidate strains have been noted to cause malaise, abdominal cramps, headache, and low-grade fever in individual volunteers, leading to speculation that additional toxic factors are produced by these strains (23, 34, 35).

Phospholipases (PL) produced by pathogenic bacteria have been shown to be capable of causing intestinal secretion. Phospholipase C (PLC) from *Clostridium perfringens* is cytotoxic (38) and causes chloride secretion from rat colonic mucosa (10). Cultured intestinal epithelial cells exposed to *C. perfringens* PLC release arachidonic acid (AA) by activating intestinal cell PLA_2 (15). Purified PLA from *Salmonella newport* causes fluid accumulation and epithelial desquamation in rabbit ileal loop assays (3). While the mechanisms of PL interactions with the enterocyte have yet to be determined, PL might directly damage membranes, interfere with normal eukaryotic transmembrane signaling, or activate cellular second-messenger

pathways by enzymatic release of diacylglycerol or inositol-1,4,5-triphosphate. These latter mechanisms need not result in visible damage to the enterocyte and do not require internalization of the PL (40).

Members of the family *Vibrionaceae* produce secreted phospholipases, some of which act as hemolysins and others that act as glycerophospholipid-cholesterol acyltransferases (30, 31, 37, 39). Taniguchi et al. (36, 37) cloned and sequenced the gene encoding the thermolabile hemolysin from *Vibrio parahaemolyticus*, and Shinoda et al. (31) characterized the protein as a PLA_2/lysophospholipase. Thornton et al. (39) sequenced a gene encoding the *Aeromonas hydrophila* glycerophospholipid-cholesterol acyltransferase and noted its ability to produce alpha-hemolytic activity on blood agar. *V. cholerae* has long been known to produce an enzyme that degrades the phospholipid lecithin (phosphatidylcholine) (11). We report here the cloning, sequencing, and chromosomal mapping of a PL gene (*lec*), the identification of the protein product, and the construction of an in-frame internal deletion mutation in the gene that results in loss of function in the *V. cholerae* vaccine strain CVD 110.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. The *V. cholerae* strains used included serogroup O1 strains E7946 (El Tor biotype, Ogawa serotype), N16961 (El Tor, Inaba), 395 (classical, Ogawa), and 569B (classical, Inaba), and serogroup O139 strain AI1837 (Bengal). The *Escherichia coli* host strains used included DH5α for construction of the cosmid library, XL-1 Blue (Stratagene, La Jolla, Calif.) for subsequent transformation and cloning, and SM10 λ*pir* for conjugation of constructs into *V. cholerae*. Cloning vectors included pHC79, pBR322, pGP704, and pBluescript KS (Stratagene). Bacteria were grown in L broth and L agar, except for the egg yolk agar assay described below. The antibiotics used were ampicillin (200 μg/ml) and polymyxin B (50 U/ml). pCVD36 is a previously constructed plasmid consisting of the entire hemolysin region on an *Xho*I fragment cloned into the *Sal*I site of pBR322 (20). pJBK128 contains an *Eco*RI-*Bgl*II fragment containing *hlyA* cloned into pBR322 (21).

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Screening a *V. cholerae* genomic DNA library for lecithinase activity. A previously prepared cosmid library consisting of *V. cholerae* El Tor strain E7946 genomic DNA cloned into pHC79 in *E. coli* host strain DH5α was screened for lecithinase activity as follows. Egg yolk was aseptically separated from commercially bought eggs (Steger's Fresh, Titusville, Md.), mixed with sterile phosphate-buffered saline, and then mixed with molten tryptic soy agar at 50°C in a ratio of 1 ml of yolk suspension per 20-ml plate. Colonies expressing lecithinase activity at 24 h were picked for further analysis. Positive colonies were easily distinguished by the presence of a zone of clearing surrounding the colony.

Sequencing. Subclones of nested deletions for sequencing were generated with exonuclease III and S1 nuclease. Sequencing of both strands of the region was accomplished by the dideoxy chain termination method with a Sequenase II kit (United States Biochemicals, Cleveland, Ohio) and ^{35}S-dATP (Amersham, Arlington Heights, Ill.). Some portions of the noncoding strand were sequenced with primers prepared by the University of Maryland DNA sequencing facility. Primers prepared from the region upstream of *lec* and the previously published upstream sequence of *hlyA* (1) allowed the determination of the intervening sequence. Sequences were analyzed with programs developed by the Genetics Computer Group at the University of Wisconsin.

Chromosomal mapping. Digoxigenin-labelled probes (Genius system; Boehringer Mannheim, Indianapolis, Ind.) of a *Bgl*II fragment containing the entire *lec* gene and an *Eco*RI-*Bgl*II fragment containing the entire *hlyA* gene were used in Southern blot analyses to probe *Cla*I digests of *V. cholerae* chromosomal DNA isolated from various strains.

Identification of a protein product. French press cell lysates were prepared from 100 ml of overnight culture as previously described (18). The cell lysate was loaded onto a 10% polyacrylamide gel without β-mercaptoethanol or boiling and then subjected to polyacrylamide gel electrophoresis (PAGE). Two identical gels were prepared; one was stained with Coomassie blue and photographed, and the other was equilibrated in 30 mM Tris–0.9% NaCl (pH 8.0) for 30 min. An egg yolk suspension was prepared from aseptically prepared yolk as described above and diluted 1:20 in 1% molten agarose (Bethesda Research Laboratories, Gaithersburg, Md.) in 100 mM Tris–0.9% NaCl (pH 8.0) at 50°C. The polyacrylamide gel was then overlaid with the yolk agarose suspension, forming a bilayer gel, and incubated at 37°C for 18 h. PL activity could then be visualized in the yolk agarose as a band of white precipitation.

Construction of a deletion mutant. An internal, in-frame deletion was constructed by excising the region between the two *Bsm*I sites into the cloned *lec* gene contained in pBluescript KS, followed by a Klenow fill-in reaction and blunt-end ligation, to construct pTF2. Deletion mutants were screened for loss of lecithinase activity on egg yolk agar. The mutated gene was sequenced across the junction to confirm that an in-frame deletion had been made. The 1.4-kb construct was then cut out with *Sst*I and *Sal*I, and ligated into the *Sst*I and *Sal*I sites of suicide vector pGP704 (26).

Selection of chromosomal recombinants. The suicide vector pGP704 is a mobilizable plasmid that has an absolute requirement for the λ *pir* factor so that it can replicate as a plasmid (26). Deletion mutations in the *lec* gene were ligated into pGP704 in SM10λ*pir* and then mobilized into *V. cholerae* by conjugation. Because *V. cholerae* does not provide the necessary π protein necessary for plasmid replication, colonies that are resistant to both polymyxin B and ampicillin reflect homologous recombination of *lec* and vector sequences into the chromosome. A second recombination event between the flanking sequences resulted in the loss of the plasmid vector, signified by a loss of ampicillin resistance. The allelic exchange was confirmed by observation of a loss of lecithinase activity and by DNA blotting.

Lecithinase activity as determined in a gel diffusion assay. Samples were grown overnight in Luria broth with or without appropriate antibiotics and centrifuged, and the pellet was resuspended in 30 mM Tris (pH 8.0)–0.9% NaCl. The cells were lysed by sonification and centrifuged, and 75 μl of supernatant was added to wells made in agarose plates containing 1% agarose, 100 mM Tris (pH 8.0), 0.9% NaCl, and 5% egg yolk suspension (egg yolk-to-PBS ratio, 1:1).

Screening of *V. cholerae* lecithinase activity with HT29/C1 cells. The toxin of *Bacteroides fragilis* is associated with diarrheal disease and can be detected by using the cultured human intestinal cell line HT29/C1 (45). This enterotoxin causes specific and striking morphological changes in these cells, including loss of cell-to-cell attachments, rounding, and swelling. The *V. cholerae* strains to be tested were grown overnight at 37°C in Luria broth; ampicillin was added at 100 μg/ml as needed to maintain plasmids. Culture supernatants were filtered through 0.45-μm-pore-size filters, and cell-free filtrates were tested in HT29/C1 cells as described previously (27). In brief, serial dilution of culture filtrates were added to subconfluent cells grown on eight-well slides. The cells were examined after 3 and 20 h, and areas of altered cell morphology or cell loss were marked as positive.

Rabbit ligated ileal loop assays with CVD 110 and CVD 113. Rabbit ligated ileal loop assays were conducted with 2-kg male New Zealand White rabbits as described by Nishibuchi et al. (28). Diluted overnight cultures (adjusted to 10^6/ml) and 4-h cultures (adjusted to 10^9/ml) of CVD 110 and CVD 113 were injected into 8-cm ligated ileal loops. Fluid accumulation in loops was measured at 18 h. Two rabbits were used for each of the two dilutions, for a total of four rabbits.

Nucleotide sequence accession number. The sequence reported in this paper has been deposited in the GenBank database under accession no. U50074.
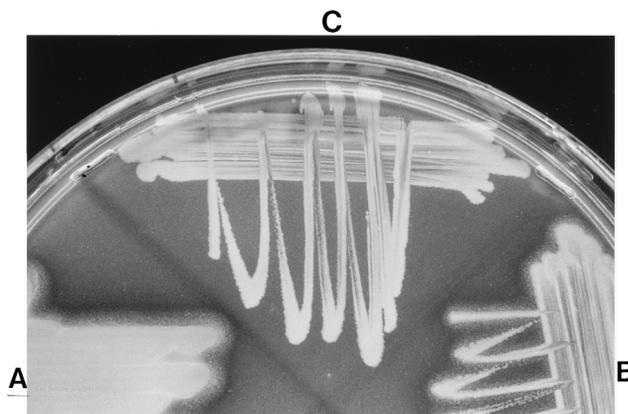

FIG. 1. Demonstration of egg yolk agar clearing by bacterial strains expressing *V. cholerae* lecithinase. (A) *V. cholerae* O1 El Tor strain E7946; (B) *E. coli* strain XL-1 Blue expressing *lec*; (C) XL-1 Blue.

RESULTS

Cloning and sequencing of *lec*. All toxigenic and vaccine strains of *V. cholerae* serogroups O1 and O139 tested were positive for lecithinase activity by the egg yolk agar assay (data not shown). The assay was then used to screen a genomic cosmid library of *V. cholerae* constructed in *E. coli*. A single colony expressing strong lecithinase activity was designated pTF1 and used in subsequent studies (Fig. 1). Two other colonies expressed weak activity on yolk agar plates. The cosmid was extracted by alkali lysis and purified. Mapping of pTF1 revealed several *Bgl*II fragments which were subcloned into the *Bam*HI site of pBluescript KS, and a clone containing a 2.2-kb *Bgl*II fragment encoding lecithinase activity was identified and designated pTF1.4.

Sequencing of the pTF1.4 insert revealed a 1,254-bp ORF designated *lec*, encoding a 418-amino-acid protein with a predicted molecular mass of 47.6 kDa (Fig. 2). The first 19 amino acids are typical of a signal peptide, as described by von Heijne (43), consistent with the secreted nature of lecithinase in *V. cholerae*. Preceding the coding region is a potential Shine-Dalgarno sequence, along with potential promoter sequences.

A homology search of the GenBank/EMBL sequence data bank revealed striking homologies between the predicted protein sequence of the *lec* gene product and phospholipases of related species. The thermolabile hemolysin (also referred to as lecithin-dependent hemolysin) from *Vibrio parahaemolyticus* shares 63.2% identity and 77.4% similarity with the protein encoded by *lec* and is also 418 amino acids long. The glycerophospholipid-cholesterol acyltransferases from *Aeromonas salmonicida* and *Aeromonas hydrophila*, two other members of the family *Vibrionaceae*, share 27.9 and 26.3% identity with the protein encoded by *lec*. These proteins are capable of erythrocyte hemolysis, although none has been shown to be critical for pathogenesis (36, 39).

Thornton et al. (39) identified a highly conserved lipid binding site present in the glycerophospholipid-cholesterol acyltransferase of *A. hydrophila*. A similar sequence exists in the *lec* gene product, including an identical 6-amino-acid region surrounding a serine residue that is critical for lipid binding in other similar enzymes (30). As noted by Thornton et al. (39), this lipid binding motif can be demonstrated in lipid binding proteins produced from many different sources, including rat hepatic lipase, porcine pancreatic lipase, and human lecithin-cholesterol acyltransferase. *V. parahaemolyticus* thermolabile

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  1      aacctagggaagccgctgggtgttttcattgaccagatgaatacctcattcacaatacccacagatctgaccagatcctcactctgattactcaatgata
                                -35                      -10

101      agaagtaaacgatgaaaaaaagactctctattctaatcgctggcttagctagcttatcggttaatgccgccactgaaccatgggcttcccctgaggcaga
                      M  K  K  R  L  S  I  L  I  A  G  L  A  S  L  S  V  N  A  A  T  E  P  W  A  S  P  E  A  E

201      agtgcttagccgcgcgcaaattcaacaagtacaaggtaaacaaacctataccatgtgcgttgttggtatcgtcctgcagccactcatgatgacccctac
          V  L  S  R  A  Q  I  Q  Q  V  Q  G  K  Q  T  Y  T  Y  V  R  C  W  Y  R  P  A  A  T  H  D  D  P  Y

301      accacttgggagtgggcaaagaatgcggatggtagctattacactattcagggttattggtggtcgagtattcgacaaaaaaacatgtttttacacgacag
          T  T  W  E  W  A  K  N  A  D  G  S  Y  Y  T  I  Q  G  Y  W  W  S  S  I  R  Q  K  N  M  F  Y  T  T  V

401      ttcagccagaaaccctgttagaacgctgtgaagagacgcttggtgtcaaccacgattttgccgatattacctatttcgcggccgatcatcgctttttcata
          Q  P  E  T  L  L  E  R  C  E  E  T  L  G  V  N  H  D  F  A  D  I  T  Y  F  A  A  D  H  R  F  S  Y

501      caaccacactatttggagcaatgatccggaggttcagtccaaccgcatcagcaaagtgatcgcgtttggcgatagcctttccgatacgggcaacattttc
          N  H  T  I  W  S  N  D  P  E  V  Q  S  N  R  I  S  K  V  I  A  F  G  D  S  L  S  D  T  G  N  I  F

601      aatgcctcacagtggcgcgcttccccaaccctgactcatggttcctaggccatttctcgaatggattcgtctggactgaatacctagcacaaggtttgaatg
          N  A  S  Q  W  R  F  P  N  P  D  S  W  F  L  G  H  F  S  N  G  F  V  W  T  E  Y  L  A  Q  G  L  N  V

701      tacctctgtataactgggcggtcggtggcgcggcagggcgtaaccagtatgtggcattaacgggcgtttatgagcaggtcagctcttatctgtcttatat
           P  L  Y  N  W  A  V  G  G  A  A  G  R  N  Q  Y  V  A  L  T  G  V  Y  E  Q  V  S  S  Y  L  S  Y  I

801      cgcactggctaaaaactatcagccagaaaacagcttgtttaccttggaatttggtctgaacgactttatgaactacaaccgttccttggccgatgtgaaa
          A  L  A  K  N  Y  Q  P  E  N  S  L  F  T  L  E  F  G  L  N  D  F  M  N  Y  N  R  S  L  A  D  V  K

901      gccgattatagttccgcgctgatccgtcttattgatgcgggagcaaaaaatcttgtcctgatgaccttgccggacgcaacgcgcgcgccacagtttcaat
          A  D  Y  S  S  A  L  I  R  L  I  D  A  G  A  K  N  L  V  L  M  T  L  P  D  A  T  R  A  P  Q  F  Q  Y

1001     atgccacgcaagagcaaattgatactgtacgcagcaaaatcatcggcatgaatgcctttatccgtgagcaagcacgttactaccaaatgcaaggtattcg
           A  T  Q  E  Q  I  D  T  V  R  S  K  I  I  G  M  N  A  F  I  R  E  Q  A  R  Y  Y  Q  M  Q  G  I  R

1101     catcgtactgtttgatgcttataccctgtttgacagtatcactactcagccagagcaacatggatttgccaacgcgaactccccttgcttagatattcga
           I  V  L  F  D  A  Y  T  L  F  D  S  I  T  T  Q  P  E  Q  H  G  F  A  N  A  N  S  P  C  L  D  I  R

1201     cgcaactctgccgccgattacctattgtcacattcactctctgcgggagtgcgccaagcaaggctcagatcgctttgtgttttgggaagtgactcatccca
          R  N  S  A  A  D  Y  L  L  S  H  S  L  S  A  E  C  A  K  Q  G  S  D  R  F  V  F  W  E  V  T  H  P  T

1301     ccaccgcgattcatcactatcttgcagagcagatttttggccaccgaaatggcacaatttccgctttaatgctcaccgtatcctgaggatggcgtaaatca
           T  A  I  H  H  Y  L  A  E  Q  I  L  A  T  E  M  A  Q  F  P  L  *

1401     tcctcagtctcacagcttagtcatcggtttctttgctttattgagcatctcttcatagccttataaaaaatgatgagatgac 1482
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FIG. 2. Nucleotide sequence of the *lec* gene. Potential −10 and −35 sites are underlined. The potential ribosome binding site and 3′ inverted repeat are doubly underlined. The putative signal sequence in the predicted protein is also underlined.

hemolysin shares this homology, and the region of the molecule with the highest degree of homology to the *lec* gene product encompasses the putative lipid binding motif, with 21 consecutive identical amino acids. A *Vibrio mimicus* arylesterase (30) and the *A. salmonicida* glycerophospholipid acyltransferase (39) contain a similar site (Fig. 3).

Linkage of *lec* and *hlyA*. A previously constructed plasmid containing the entire hemolysin region, pCVD 36, was noted to contain a restriction map similar to the *lec* region upstream from the *hlyA* region. A *Cla*I digest of pCVD36, when probed with digoxigenen-labelled probes consisting of the *Bgl*II fragment containing *lec* and the *Eco*RI-*Hin*dIII fragment containing *hlyA*, hybridized to the same 9.6-kb fragment. *Cla*I chro-

mosomal digests of all toxigenic *V. cholerae* strains tested, including classical biotype strains 395 and 569B, El Tor strains E7946 and N16961, and O139 (Bengal) strain AI1837, possessed similar-sized fragments with homology to the *lec* and *hlyA* probes, indicating that this region is conserved across toxigenic strain lines.

Sequence analysis of the region between *lec* and *hlyA* revealed the intervening region to be 970 bp long, with no obvious open reading frame. The two genes are transcribed in opposite directions (Fig. 4). Interestingly, a 128-bp section of the intervening sequence, upstream from and on the same strand with *lec*, shares 78.9% identity with the gene *viuA*, which encodes the vibriobactin receptor (6). This iron-regulated outer membrane protein binds the siderophore vibriobactin to the bacterial cell surface. However, no obvious open reading frame is present in the DNA sequence homologous to *via*.

Identifying the *lec* gene product. A protein with lecithinase activity is present in French press cell lysates of *E. coli* transformed with pTF1.4, as shown by the PAGE-egg yolk agarose overlay results. A single dense white band of precipitation is seen at approximately 45 kDa and is absent from XL-1 Blue containing vector sequences (Fig. 5). This finding agrees with

V. cholerae lecithinase	K	V	I	A	F	G	D	S	L	S
V. parahaemolyticus thermolabile hemolysin	K	V	V	A	L	G	D	S	L	S
A. hydrophila acyltransferase	R	I	V	M	F	G	D	S	L	S
V. mimicus arylesterase	K	L	L	V	L	G	D	S	L	S
Rat hepatic lipase	S	V	H	L	F	I	D	S	L	Q
Human lipoprotein lipase	S	I	H	L	F	I	D	S	L	L
Human lecithin cholesterol acyltransferase	P	V	F	L	I	G	H	S	L	G

FIG. 3. Putative lipid-binding domain.
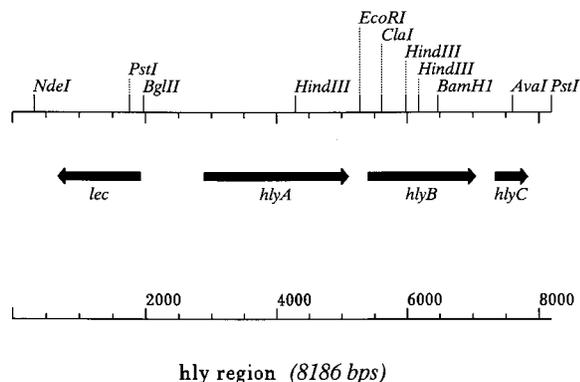
FIG. 4. Restriction map of the hemolysin/lecithinase region.

FIG. 6. Mutation of *lec* results in loss of lecithinase activity on egg yolk agar. Complementation of *V. cholerae* carrying the *lec* mutation with pTF1.4 results in restoration of lecithinase activity. 1, DH5α (pBluescript KS); 2, CVD 113 (pCVD36); 3, CVD 113 (pBluescript KS); 4, pCVD 113; 5, CVD 113(pTF1.4); 6, DH5α(pTF1.4); 7, CVD 110.

the expected size of the *lec* gene product, especially if the signal sequence is cleaved off as predicted. French press cell lysates of wild-type classical *V. cholerae* 395 show a similar although less dense band of precipitation, also at approximately 45 kDa (data not shown). The strong exogenous promoter present in pTF1.4 presumably results in the production of large amounts of lecithinase, whereas optimal conditions for *V. cholerae* lecithinase production remain undetermined, and this may account for the difference in band density seen.

Construction of a *lec* mutant. An in-frame *lec* mutation (CVD 113) was constructed in CVD 110, a previously constructed El Tor vaccine candidate in which the entire virulence cassette (*ctxA*, *zot*, and *ace*) is deleted and the hemolysin gene (*hlyA*) is mutated (25). Loss of lecithinase activity on egg yolk agar confirmed that wild-type *lec* had been replaced by the internally deleted *lec*. This also served to demonstrate that a second gene encoding a lecithinase capable of producing a zone of clearing on egg yolk agar is not present in CVD 110. Loss of the internal deletion fragment was confirmed by Southern blotting of CVD 110 chromosomal DNA with using the

digoxigenen-labelled *Bsm*I fragment as a probe (data not shown). Complementation of this mutant with pTF1.4 restored lecithinase activity on egg yolk agar (Fig. 6).

***V. cholerae* lecithinase activity on HT29/C1 cells.** Addition of CVD 110 supernatant caused cytotoxicity and cell loss at a 1/200 dilution at 3 h and at a 1/400 dilution at 20 h. CVD 113, CVD 113 transformed with plasmid vector pBluescript, and CVD 113 transformed with pTF1.4 did not cause HT29/C1 cell loss even at dilutions as high as 1/4.

Rabbit ligated ileal loop assays with CVD 110 and CVD 113. The mean fluid accumulation ratio in nine rabbit ligated ileal loops injected with CVD 110 was not significantly different from that in nine loops injected with CVD 113 (0.76 and 0.63 ml/cm, respectively; $P = 0.7$).

DISCUSSION

In this report, we describe the cloning and sequencing of a *V. cholerae* gene encoding a PL. The predicted protein sequence contains a putative lipid binding motif that was previously demonstrated in other bacterial and eukaryotic PLs and shared striking overall sequence similarity with sequences of other PLs produced by members of the *Vibrionaceae*. The *lec* gene was located upstream of the *hlyA* gene encoding the El Tor hemolysin. In addition to *hlyA* and *lec*, the 9.6-kb *Cla*I fragment conserved among all *V. cholerae* strains tested contains *hlyC*, which encodes a lipase whose sequence is similar to that of lipases found in *Pseudomonas* species (7). In the suckling-mouse model, loss of *hlyC* resulted in diminished virulence and colonization ability, and the authors conclude that the lipase may help break down the mucus layer that protects gut epithelium, thus enhancing colonization ability. The secreted hemolysin encoded by *hlyA* is enterotoxic and cytotoxic (2, 16), although inactivation of the gene as measured by loss of hemolytic activity did not further attenuate El Tor vaccine strains (20, 23). Conservation among different toxigenic *V. cholerae* serogroups and biotypes of the lecithinase-hemolysin-lipase locus suggests that lecithinase may be a component of a group of enzymes whose function is to damage enterocyte cell mem-
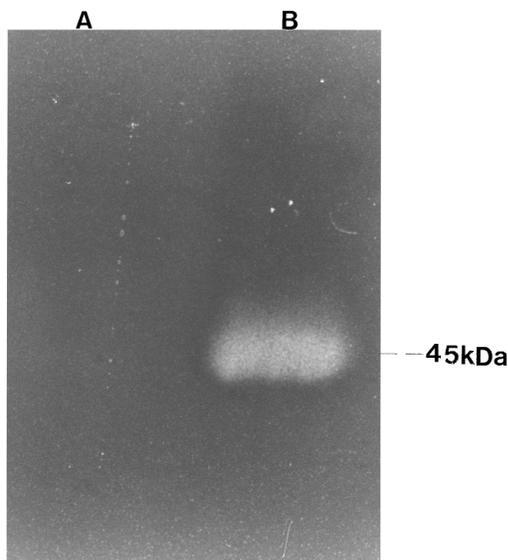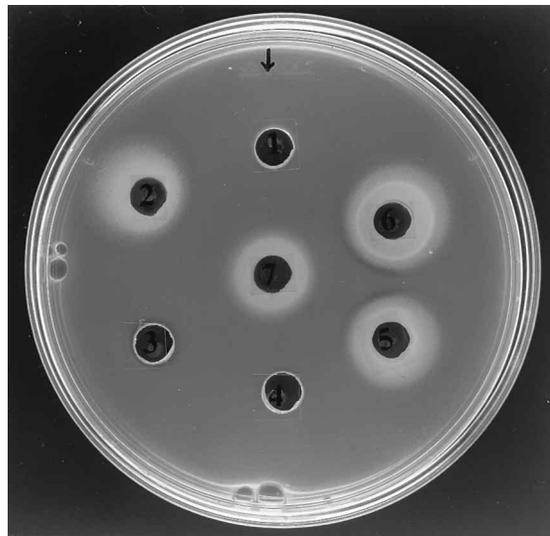

FIG. 5. Demonstration of lecithinase activity in sodium dodecyl sulfate-PAGE of French press preparations of *E. coli* containing vector sequences (A) and pTF-1 (B). A band of precipitation is seen at approximately 45 kDa in an overlay of egg yolk agarose.

branes. Whether this region is coordinately regulated, perhaps as part of the iron-sensing system that controls hemolysin and vibriobactin production (14), remains to be investigated. The existence in the *V. cholerae* chromosome of a pathogenicity island encoding *tcp*, *acf*, and *toxT* (5), as well as the linkage of *ctx*, *zot*, and *ace* (41) on a bacteriophage (44), has previously been established.

An in-frame deletion mutation of the *lec* gene was constructed and recombined into the chromosome of attenuated *V. cholerae* CVD 110. The resulting strain, CVD 113, demonstrated no lecithinase activity on egg yolk agar, but activity was restored by complementation with the cloned *lec* gene. Initial experiments to establish a potential role for lecithinase in the pathogenesis of disease due to *V. cholerae* utilized cultured intestinal epithelial cells and an in vivo ligated intestinal loop assay. The HT29/C1 intestinal cell line has been used to detect the enterotoxin of enterotoxigenic *B. fragilis* strains associated with diarrheal disease (45). In this system, cell detachment and altered cell morphology correlate with the activity of the *B. fragilis* enterotoxin. Culture supernatants of CVD 110 caused cell loss, whereas supernatants of the *lec* mutant CVD 113 did not cause these changes. CVD 113 was also tested in ligated rabbit ileal loops, a classic method for detecting enterotoxins. However, in this in vivo system, deletion of lecithinase activity did not appear to significantly diminish the ability of CVD 110 to cause fluid accumulation. This result suggests that fluid accumulation in rabbit intestinal loops in intact animals is not caused by lecithinase activity.

PLs have been implicated in the pathogenesis of a number of bacterial infections. Unlike many other toxins, they do not require eukaryotic cell internalization to interfere with cellular function. By interacting with the eukaryotic cell membrane, PLs are capable of interrupting or altering normal cellular physiology via second-messenger systems. For example, a PLC produced by *Staphylococcus aureus* releases glycosyl phosphatidylinositol-anchored membrane surface proteins (9, 24), potentially disrupting membrane signaling pathways. PL digestion of membrane phospholipids liberates into the cytoplasmic milieu a variety of potent intracellular messenger substances, including diacylglycerol and inositol-1,3,5-triphosphate, that may in turn activate phosphokinase C or calcium gating systems (40). Furthermore, diacylglycerol is converted by intracellular diacylglycerol lipase to AA, the precursor of the inflammation-modulating eicosanoids. Enterocyte membranes exposed to PL may also release inflammatory mediators such as AA into the gut lumen, and AA metabolites such as prostaglandin E_2 have been shown to cause intestinal secretion (29). Other perturbations of cellular physiology are also possible. *C. perfringens* PLC causes vasoconstriction in the isolated rat aorta via an AA pathway (12) and stimulates the formation of platelet-activating factor in cultured intestinal epithelial cells (19). A PL produced by *Bacillus cereus* induces interleukin-2 receptor expression in cultured mouse lymphocytes (17). These mechanisms of action would be consistent with the histopathological observation that minimal epithelial damage is seen even in cases of severe diarrhea due to *Vibrio* infection.

Cholera has often been considered a noninflammatory disease; however, both recent and historical evidence suggests that infection in humans causes mild intestinal inflammation. Several histopathological studies of patients naturally infected with *V. cholerae* have noted signs of intestinal inflammation, including the presence of leukocytes in stool samples (33) and an influx of neutrophils into the intestinal epithelium (13). Fecal lactoferrin, a marker of intraluminal intestinal neutrophils, shows increased levels in stool samples from volunteers experimentally infected with *V. cholerae* El Tor (32), while C-reactive protein and leukocyte counts are elevated in the blood of cholera patients (4). Intravenous infusion of indomethacin, a prostaglandin synthetase inhibitor, decreases jejunal fluid secretion and luminal release of prostaglandin E_2 in naturally acquired disease (42). Enzymes such as PLs might trigger inflammatory cascades via AA, leading to the production of neutrophil chemoattractants such as leukotrienes. While the major feature of natural infection, voluminous diarrheal purging, is clearly mediated by cholera enterotoxin, the absence of *ctxA* in live vaccine strains is insufficient to abolish mild diarrhea in some patients. Whether this mild diarrhea is due to intestinal inflammation or involves a PL in any capacity is not known.

CVD 103-HgR is a well-tolerated live, oral vaccine strain that is effective in the prevention of infection with *V. cholerae* (22). However, the vaccine strains derived from some other strains remain reactogenic (34, 35), although the severe diarrhea characteristic of infection with toxigenic strains is not seen. Stool samples taken from volunteers who have ingested the El Tor vaccine strain CVD 110 suggest that at least some degree of inflammation, as measured by the neutrophil specific granule marker lactoferrin, is engendered by this vaccine strain (32). While the genes encoding cholera enterotoxin A subunit (*ctxA*), zonula occludens toxin (*zot*), accessory cholera enterotoxin (*ace*), and hemolysin (*hlyA*) have all been deleted or mutagenized in recent vaccine strains, *lec* is still present. No evidence exists to suggest that *lec* contributes to the reactogenicity of vaccine strains, and our initial experiments with a *lec* mutant in a ligated intestinal loop model did not support a role for lecithinase in fluid secretion. However, the proximity of *lec* to the hemolysin region on the chromosome and the known effects of PLs in other bacterial infections suggest that further characterization of the role of PL in *V. cholerae* infection is warranted.

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Editor: P. E. Orndorff