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Cloning and Expression of the Phospholipase C Gene from *Clostridium perfringens* and *Clostridium bifermentans*

J. YUN TSO* AND CHRISTIAN SIEBEL†

Protein Design Labs, Inc., 3181 Porter Drive, Palo Alto, California 94304

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The phospholipase C gene from *Clostridium perfringens* was isolated, and its sequence was determined. It was found that the structural gene codes for a protein of 399 amino acid residues. The NH_2-terminal residues have the typical features of a signal peptide and are probably cleaved after secretion. *Escherichia coli* cells harboring the phospholipase C gene-containing plasmid expressed high levels of this protein in the periplasmic space. Phospholipase C purified from *E. coli* transformants was enzymatically active, hemolytic to erythrocytes, and toxic to animals when injected intravenously. The phospholipase C gene from a related organism, *Clostridium bifermantans*, was also isolated. The two phospholipase C genes were found to be 64% homologous in coding sequence. The *C. bifermentans* protein, however, was 50-fold less active enzymatically than the *C. perfringens* enzyme.

Clostridium perfringens phospholipase C (phosphatidylcholine cholinephosphohydrolase, EC 3.1.4.3) catalyzes the hydrolysis of lecithin into phosphorylcholine and 1,2-diacylglyceride (15). In addition to its enzymatic activity, this protein is also known to have lethal, hemolytic, and necrotic activities (15). Phospholipase C is actively secreted into the medium by *C. perfringens* during cell growth and may play an important role in the pathogenesis of gas gangrene. Although the protein (molecular weight, 43,000) has been purified (13, 23, 25) and much research has been conducted on its various activities, little is known about its structure or relationship with other bacterial phospholipase Cs, many of which are nonhemolytic and nontoxic. Furthermore, it has been difficult to study the activities of phospholipase C with preparations which are often contaminated with other clostridial hydrolytic enzymes and toxins. In this paper, we report the cloning and overexpression of the *C. perfringens* phospholipase C gene in *Escherichia coli* as a first step toward a better understanding of the structure and function of the protein. For comparison, we also cloned and sequenced a related phospholipase C gene from *Clostridium bifermentans*.

MATERIALS AND METHODS

Isolation of nucleic acids. DNA was isolated from *C. perfringens* ATCC 13124 or *C. bifermentans* ATCC 638 by the lysozyme-sodium dodecyl sulfate (SDS)-proteinase K method. Cells were grown in 1 liter of Todd-Hewitt broth at 37°C without shaking to stationary phase, harvested by centrifugation, and suspended in 4 ml of TE buffer (10 mM Tris hydrochloride [pH 8.0], 5 mM EDTA) containing 2 mg of lysozyme per ml. After incubation at 37°C for 1 h, SDS was added to 0.5% (wt/vol) and EDTA was added to 50 mM. The lysed cell mixture was then treated with proteinase K (100 μg/ml) at 60°C for 4 h, phenol extracted, and ethanol precipitated. DNA was spooled from the ethanol precipitates and resuspended in TE buffer. Plasmid DNA was prepared by the procedure of Birnboim and Doly (4), and recombinant bacteriophage DNA was prepared by the plate lysate method of Fritsch (8).

Preparation of *C. perfringens* and *C. bifermentans* DNA libraries. *C. perfringens* chromosomal DNA was digested with either *Hin*dIII or *Eco*RI and cloned into the respective sites in the plasmid pEMBL8+ (6). The *Hin*dIII- and *Eco*RI-generated plasmids were then used to transform *E. coli* host DH1 (10), and transformants were plated on LB agar plates containing 50 mg of ampicillin per ml and 1% (vol/vol) sheep erythrocytes.

The first *C. bifermentans* DNA library was generated by digestion of the chromosomal DNA with *Eco*RI and cloned into the *Eco*RI site of λgt10 (11). The genomic library was plated on *E. coli* host C600 Hfl and screened by plaque hybridization (1), using the cloned *C. perfringens* phospholipase C gene as a probe. Hybridization was performed with 5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–50% formamide–1× Denhardt solution–0.2% SDS–0.1 mg of calf thymus DNA per ml at 30°C overnight. The second *C. bifermentans* DNA library was generated by cloning *Hin*dIII-digested chromosomal DNA into the *Hin*dIII site of pUC19. *E. coli* DH1 transformants containing the recombinant plasmids were then screened by colony hybridization (9), using the truncated *C. bifermentans* phospholipase C gene isolated from the first library as a probe.

DNA sequence analysis. DNA sequencing was performed by the deoxynucleotide chain termination technique of Sanger et al. (20).

Purification of phospholipase C from *C. perfringens*, *E. coli* DH1(pPLC8), and *E. coli* DH1(pCBPLC3). *C. perfringens* ATCC 13124 was grown according to the procedure of Krug and Kent (13). Cells were harvested by centrifugation, and the spent medium was brought to 50% saturation by addition of solid $(NH_4)_2SO_4$. The $(NH_4)_2SO_4$ precipitate was collected by centrifugation and suspended in a minimum volume of buffer C (75 mM Tris hydrochloride [pH 8.0], 5% ethanol, 2 mM $CaCl_2$) before being desalted on a Sephadex G-50 column. The desalted $(NH_4)_2SO_4$ fraction was then passed through a DEAE-Sepharose column to remove some of the contaminant proteins. The DEAE-Sepharose flow-through fraction was dialyzed against 20 mM bis(2-hydroxyethyl)–Tris hydrochloride (pH 6.0), loaded onto a Q-Sepha-

* Corresponding author.
† Present address: Whitehead Institute, Cambridge, MA 02142.

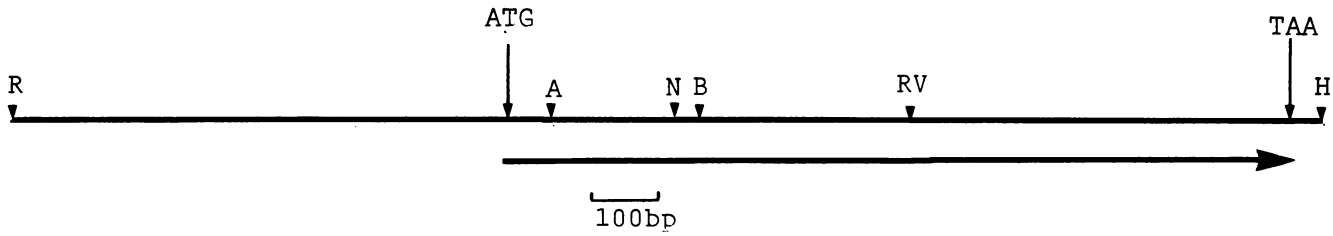

FIG. 1. Restriction map of the 2.0-kbp *Eco*RI-*Hin*dIII fragment in pPLC8. →, Open reading frame. ATG and TAA indicate the positions of the start and stop codons, respectively. Symbols for restriction enzymes: A, *Acc*I; B, *Bam*HI; H, *Hin*dIII; N, *Nde*I; R, *Eco*RI; and RV, *Eco*RV.

rose (Pharmacia Fine Chemicals, Piscataway, N.J.) column, and eluted with a step gradient of 50, 125, and 250 mM NaCl in 20 mM bis(2-hydroxyethyl–Tris hydrochloride (pH 6.0) (7). Phospholipase C eluted at 250 mM salt was at least 98% pure on the basis of SDS-polyacrylamide gel electrophoresis analysis. The overall yield was 0.5 mg per liter of culture.

E. coli DH1(pPLC8) and DH1(pCBPLC3) were grown in M9 medium supplemented with 0.4% (wt/vol) Casamino Acids (Difco Laboratories, Detroit, Mich.), 0.2% (wt/vol) glucose, and 50 mg of ampicillin per ml at 37°C to an optical density at 650 nm of 0.9. Cells were harvested by centrifugation and subjected to osmotic shock treatment to isolate proteins located in the periplasmic space (18). The osmotic shock fluid was then dialyzed against 20 mM bis(2-hydroxyethyl)–Tris hydrochloride (pH 6.0) before being loaded onto a Q-Sepharose column. Phospholipase C was eluted as described above. For the DH1(pPLC8) preparation, the protein was at least 90% pure, with an overall yield of 10 mg per liter of culture. For the DH1(pCBPLC3) preparation, the Q-Sepharose fraction was dialyzed against 50 mM Tris hydrochloride (pH 7.8)–2 mM $CaCl_2$–5% ethanol before being loaded onto a DEAE-Sepharose column and was eluted with a 100 and 150 mM NaCl step gradient in the same buffer. The *C. bifermentans* phospholipase C eluted at 150 mM NaCl and was 80% pure. Overall yield for this protein was 2 mg per liter of culture.

Analytical methods. Phospholipase C activity was determined by the alkaline phosphatase-coupled assay described by Krug and Kent (13), using an ethanoic dispersion of phosphatidylcholine substrate. One unit of phospholipase C activity equals one micromole of product formed per minute at 37°C. Hemolytic activity of phospholipase C was determined with sheep erythrocytes as described by Takahashi et al. (25), with modifications. Erythrocytes were washed and suspended at 1% (vol/vol) in an isotonic borate buffer (pH 7.6) consisting of 0.57 g of $Na_2B_4O_7 \cdot 10H_2O$, 2.1 g of H_3BO_3, 1.8 g of $CaCl_2 \cdot 2H_2O$, and 7.5 g of NaCl per liter. When diluted 1:4 with distilled water, this suspension gave an optical density at 550 nm of 0.5, corresponding to 50% hemolysis. The reaction mixture contained 0.15 ml of serial dilutions of enzyme in borate buffer containing 0.1 mg of bovine serum albumin per ml and 0.15 ml of the erythrocyte suspension. The mixture was incubated for 30 min at 37°C and then for 30 min at 4°C for hot-cold hemolysis. After centrifugation, the degree of hemolysis in the supernatant fluid was measured spectrophotometrically at 550 nm. One 50% hemolysis unit is defined as the amount of enzyme that causes 50% hemolysis in 0.3 ml of borate buffer under the conditions described above.

Protein was assayed as described by Bradford (5), using bovine serum albumin for standard protein concentrations.

Toxicity tests. Phospholipase C preparations in Hanks salt solution containing 0.1% (wt/vol) gelatin were injected in 0.25-ml volumes into the tail veins of 8-week-old, 18- to 22-g mice.

RESULTS

Isolation of the *C. perfringens* phospholipase C gene. *C. perfringens* chromosomal DNA was digested with either *Hin*dIII or *Eco*RI , cloned into the respective sites in plasmid pEMBL8+, and used to transform *E. coli*. After 2,000 transformants from each library were screened on LB agar plates containing ampicillin and erythrocytes, five colonies that created zones of hemolysis were isolated. All five isolates exhibited phospholipase C activity in the periplasmic space as assayed by the method of Krug and Kent (13). Restriction analysis of the two kinds of plasmid (*Hin*dIII- and *Eco*RI-generated recombinants) in these colonies indicated that they shared a common 2.0-kilobase-pair (kbp) *Eco*RI-*Hin*dIII fragment in their inserts. This 2.0-kbp *Eco*RI and *Hin*dIII fragment was then subcloned between the *Eco*RI and *Hin*dIII sites of pEMBL8+ for detailed restriction and DNA sequence analysis (20). *E. coli* transformants harboring the resulting plasmid, pPLC8, were hemolytic and exhibited phospholipase C activity in the periplasmic space, confirming that the gene was intact in this 2.0-kbp fragment. Furthermore, a predominant protein with molecular weight of 43,000 was present in the osmotic shock fluid (18) from the periplasmic space of DH1(pPLC8). This protein was absent from the shock fluid of the host cell. The 2.0-kbp *Eco*RI-*Hin*dIII fragment was also subcloned into pEMBL9 (6) to generate plasmid pPLC9. *E. coli* transformants harboring pPLC9 still expressed the 43,000-dalton protein in the periplasmic space. This result indicated the *Clostridium* promoter could direct the synthesis of this protein, disregarding the gene orientation with respect to the *E. coli lacZ* promoter in the vector.

The restriction map of the 2.0-kbp *Eco*RI-*Hin*dIII fragment is shown in Fig. 1. Sequence analysis (20) of this fragment revealed an open reading frame of 1,196 bp located in the *Hin*dIII half of the fragment (Fig. 1 and 2). The first 22 amino acid residues of this protein have the typical features of a secretory signal peptide: a positively charged NH_2-terminal region and a hydrophobic central section, followed by two small nonpolar residues with one residue between them. Thus, the signal peptidase would cleave after Ala-22, giving a mature protein of 43,179 daltons. The amino acid composition of the deduced mature protein sequence agrees very well with that of the purified *C. perfringens* phospholipase C (13). Taken together, these results show unequivocally that the *C. perfringens* phospholipase C gene was cloned and expressed in *E. coli*.

Isolation of the phospholipase C gene from *C. bifermentans*. *C. bifermentans* secretes an α-toxin antigenically related to

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                                                                  30                                                                  60
(1) ATG AAA AGA AAG ATT TGT AAG GCG CTT ATT TGT GCC GCG CTA GCA ACT ACG CTA TGG GCT
(2)         TG   A GC   TA   A AAA G A TC  AA  ATA CTG TGC  T  TTA GGT T    T A
(1) Met Lys Arg Lys Ile Cys Lys Ala Leu Ile Cys Ala Ala Leu Ala Thr Thr Leu Trp Ala
(2)         Met Lys Ala Leu     Lys Val Ser Asn Ile Leu Cys Val Leu Gly     Cys Thr
                     5                   10                  15                  20

                                                                  90                                                                 120
    GGG GCA TCA ACT AAA GTC TAC GCT TGG GAT GGA AAG ATT GAT GGA ACA GGA ACT CAT GCT
    CTT ATG GGG GG  ACT TCT  T   A       T   A  AA      G       G   A     T A
    Gly Ala Ser Thr Lys Val Tyr Ala Trp Asp Gly Lys Ile Asp Gly Thr Gly Thr His Ala
    Leu Met Gly Gly Thr Ser                         Lys                         Ser
                  25                    30                  35                  40

                                                                 150                                                                 180
    ATG ATT GTA ACT CAA GGG GTT TCA ATC TTA GAA AAT GAT CTG TCC AAA AAT GAA CAA GAA
    T A   A C GAA  T     T A AGT  G C  A C        T A AGT GG        T T C G
    Met Ile Val Thr Gln Gly Val Ser Ile Leu Glu Asn Asp Leu Ser Lys Asn Glu Pro Glu
    Leu     Ala Glu His     Leu     Met     Asn                 Gly         Ser Gln
                     45                  50                  55                  60

                                                                 210                                                                 240
    AGT GTA AGA AAA AAC TTA GAG ATT TTA AAA GAG AAC ATG CAT GAG CTT CAA TTA GGT TCT
    CAA  T  AG G T  T A T A A         T  A T  T A GGG  T T A A G      G
    Ser Val Arg Lys Asn Leu Glu Ile Leu Lys Glu Asn Met His Glu Leu Gln Leu Gly Ser
    Gln     Lys Asp     Ile Lys         Asn     Tyr Leu Gly Asp     Lys
                     65                  70                  75                  80

                                                                 270                                                                 300
    ACT TAT CCA GAT TAT GAT AAG AAT GCA TAT GAT CTA TAT CAA GAT CAT TTC TGG GAT CCT
      A               C     CCT   C                                       AT
    Thr Tyr Pro Asp Tyr Asp Lys Asn Ala Tyr Asp Leu Tyr Gln Asp His Phe Trp Asp Pro
                           Pro                                          Tyr
                     85                  90                  95                 100

                                                                 330                                                                 360
    GAT ACA GAT AAT AAT TTC TCA AAG GAT AAT AGT TGG TAT TTA GCT TAT TCT ATA CCT GAC
      C  T GA  C       T A T  TT                   GC  T A    CC      TA   T
    Asp Thr Asp Asn Asn Phe Ser Lys Asp Asn Ser Trp Tyr Leu Ala Tyr Ser Ile Pro Asp
           Gly             Thr Ile                 Ala Ser     Pro     Tyr
                    105                 110                 115                 120

                                                                 390                                                                 420
    ACA GGG GAA TCA CAA ATA AGA AAA TTT TCA GCA TTA GCT AGA TAT GAA TGG CAA AGA GGA
        A C AG  AAC TC  G       G     G T A          A  A         G   AG
    Thr Gly Glu Ser Gln Ile Arg Lys Phe Ser Ala Leu Ala Arg Tyr Glu Trp Gln Arg Gly
        Ser Arg Asn Ser Val             Ala Thr         Lys Asn         Glu Lys
                    125                 130                 135                 140
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                                                450                                                 480
AAC TAT AAA CAA GCT ACA TTC TAT CTT GGA GAG GCT ATG CAC TAT TTT GGA GAT ATA GAT
  T  T       G              CT  T A   C C A  GA T A   T     C A   G     T   A
Asn Tyr Lys Gln Ala Thr Phe Tyr Leu Gly Glu Ala Met His Tyr Phe Gly Asp Ile Asp
    Phe     Glu             Leu         Gln Gly Leu         Leu         Leu Asn
                145                 150                 155                 160

                                                510                                                 540
ACT CCA TAT CAT CCT GCT AAT GTT ACT GCC GTT GAT AGC GCA GGA CAT GTT AAG TTT GAG
                  G  T A       A       A   G       T C                 A  A    A
Thr Pro Tyr His Pro Ala Asn Val Thr Ala Val Asp Ser Ala Gly His Val Lys Phe Glu
                  Ala Ser                           Pro                     Tyr
                165                 170                 175                 180

                                                570                                                 600
ACT TTT GCA GAG GAA AGA AAA GAA CAG TAT AAA ATA AAC ACA GCA GGT TGC AAA ACT AAT
         T   A               T A C     GCT T     T   T T     A AAT G T      CA
Thr Phe Ala Glu Glu Arg Lys Glu Gln Tyr Lys Ile Asn Thr Ala Gly Cys Lys Thr Asn
        Val                 Asp Asn     Ala Leu         Ser     Asn Asp      Thr
                185                 190                 195                 200

                                                630                                                 660
GAG GCT TTT TAT ACT GAT ATC TTA AAA AAC AAA GAT TTT AAT GCA TGG TCA AAA GAA TAT
TCA  GA G A      AA   A GCT A G G    T CC  AG          AAA     ATG  C  C   A C
Glu Ala Phe Tyr Thr Asp Ile Leu Lys Asn Lys Asp Phe Asn Ala Trp Ser Lys Glu Tyr
Ser Gly Val     Lys Glu Ala Met Glu     Pro Ser          Lys     Met Thr Gln Asn
                205                 210                 215                 220

                                                690                                                 720
GCA AGA GGT TTT GCT AAA ACA GGA AAA TCA ATA TAC TAT AGT CAT GCT AGC ATG AGT CAT
T T  T  AAA  A       G  T   CT     GAT T     T             T A  CT
Ala Arg Gly Phe Ala Lys Thr Gly Lys Ser Ile Tyr Tyr Ser His Ala Ser Met Ser His
Ser Ile Lys Tyr         Ile Ala     Asp Leu                 Ser Thr
                225                 230                 235                 240

                                                750                                                 780
AGT TGG GAT GAT TGG GAT TAT GCA GCA AAG GTA ACT TTA GCT AAC TCT CAA AAA GGA ACA
                          T T  G   GA  A  G   A   AAA   T         GT  T T   T
Ser Trp Asp Asp Trp Asp Tyr Ala Ala Lys Val Thr Leu Ala Asn Ser Gln Lys Gly Thr
                          Ser Gly Arg Glu Ala Ile Lys             Val Cys
                245                 250                 255                 260

                                                810                                                 840
GCG GGA TAT ATT TAT AGA TTC TTA CAC GAT GTA TCA GAG GGT AAT GAT CCA TCA GTT GGA
  T      T  T A           A G A T   A   T   T A T   A     ACA GG  GAT AA  AT
Ala Gly Tyr Ile Tyr Arg Phe Leu His Asp Val Ser Glu Gly Asn Asp Pro Ser Val Gly
        Phe Leu             Met Asn Glu         Asn         Thr Gly Asp Asn Asp
                265                 270                 275                 280
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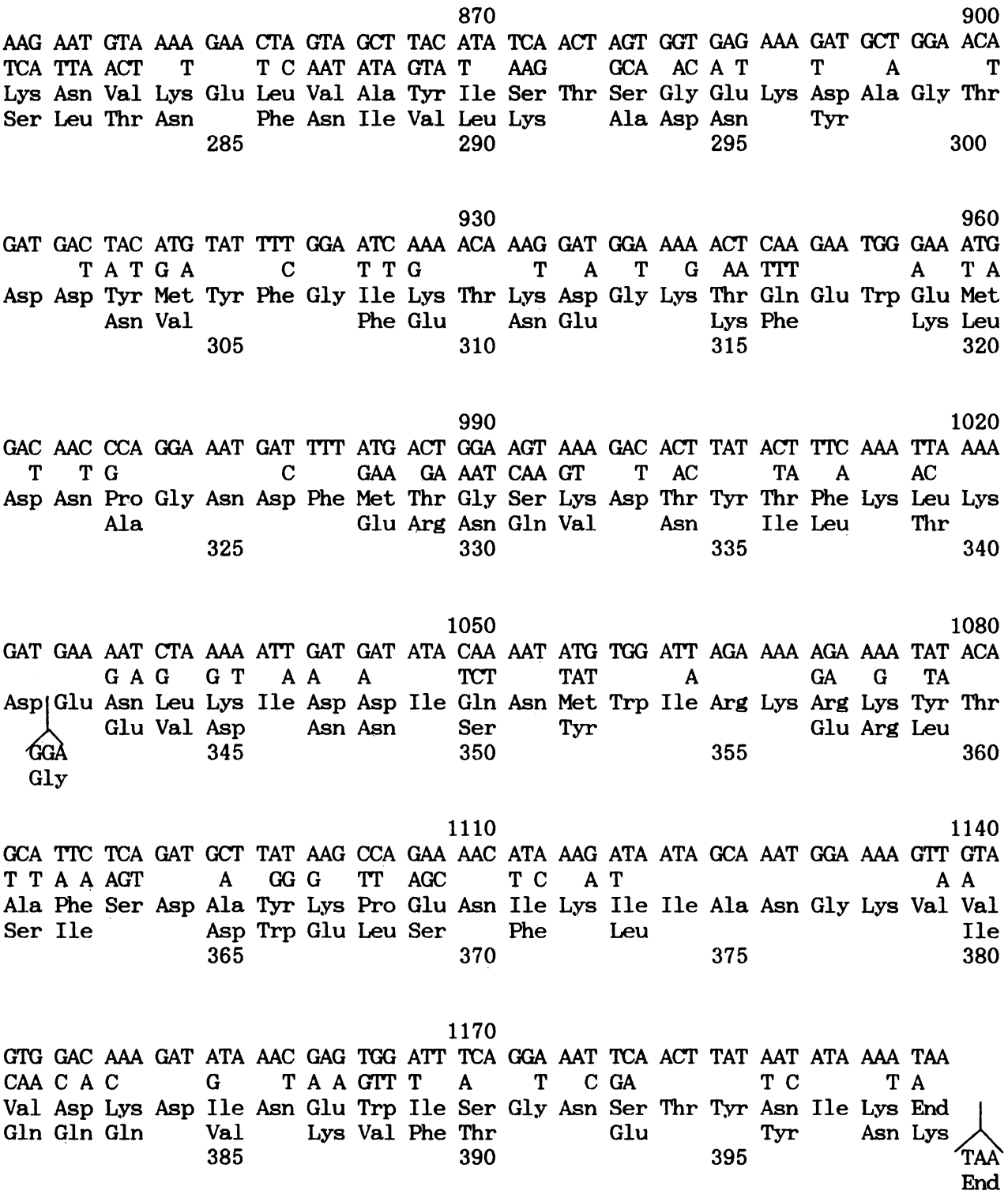

FIG. 2. Nucleotide and deduced amino acid sequences of *C. perfringens* (1) and *C. bifermentans* (2) phospholipase genes. Both the nucleotide and amino acid sequences are numbered from the ATG codon. Only nucleotides and deduced amino acids that are different from those of the *C. perfringens* gene are shown for the *C. bifermentans* gene. Indicated are nucleotide insertions (⅄) and putative processing sites of the signal peptide for the *C. perfringens* protein (↓) and for the *C. bifermentans* protein (↑).

the *C. perfringens* phospholipase C. Miles and Miles (16) reported that enzymatically equal amounts of phospholipase C from several strains of *C. bifermentans* were up to 75-fold less toxic to animals than was the corresponding amount of *C. perfringens* enzyme, which indicated that enzymatic activity might not be equivalent to toxicity for this protein. However, because of the poor yield from the culture, the *C. bifermentans* enzyme had never been purified. To better

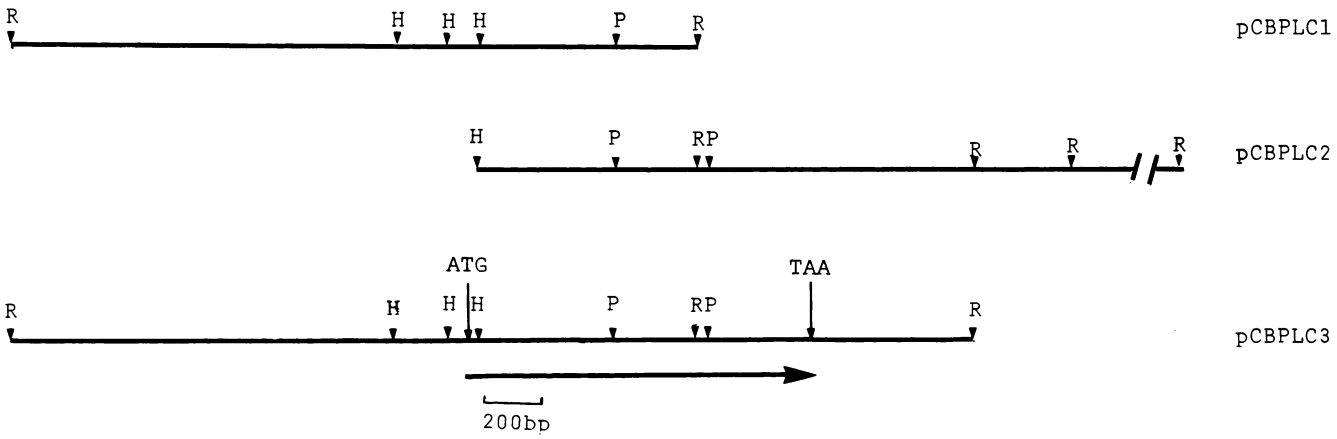

FIG. 3. Restriction maps of inserts in plasmids pCBPLC1, pCBPLC2, and pCBPLC3. Symbol and abbreviations are as in the legend to Fig. 1, with the addition of P (*PstI*).

characterize this protein, we cloned and overexpressed its gene in *E. coli*.

We used the previously cloned *C. perfringens* phospholipase C gene as a probe to screen a *C. bifermentans* genomic library cloned into the *Eco*RI site of λgt10. After 2,000 plaques were screened, 20 phages that hybridized to the probe were isolated. All of these phages contain a 2.5-kbp *Eco*RI insert which was subcloned into pEMBL8+ to form plasmid pCBPLC1. Restriction and Southern analysis (24) of the insert in pCBPLC1 indicated that the sequence related to the *C. perfringens* phospholipase C gene was located in the 0.83-kbp *Hin*dIII-*Eco*RI fragment at the left end of the insert (Fig. 3). DNA sequence analysis of this fragment confirmed that it indeed contained part of the phospholipase C-coding sequence but was truncated at the *Eco*RI site. A second genomic library was therefore created to isolate the missing coding sequence. The second library was created by cloning *Hin*dIII-digested chromosomal DNA from *C. bifermentans* into the *Hin*dIII site of pUC19. *E. coli* transformants containing the recombinant plasmids were then screened by colony hybridization (9), using the 0.83-kbp *Hin*dIII-*Eco*RI fragment from pCBPLC1 as a probe. From 2,000 transformants, seven colonies that hybridized to the probe were isolated. All plasmids in these colonies, of which a representative one was designated pCBPLC2, contained a 5.2-kbp *Hin*dIII fragment in their inserts.

Restriction maps of the inserts in pCBPLC1 and pCBPLC2 are shown in Fig. 3. The 1.0-kbp *Eco*RI fragment adjacent to the *Eco*RI insert of pCBPLC1 in the chromosome was subcloned from pCBPLC2 into pCBPLC1 to reconstitute the entire phospholipase C gene. The resulting plasmid was designated pCBPLC3 (Fig. 3). The entire coding sequence for phospholipase C in pCBPLC3 is shown in Fig. 2. The gene codes for a protein of 398 amino acid residues, with the NH_2-terminal 22 residues corresponding to a signal peptide. The mature *C. perfringens* and *C. bifermentans* phospholipase C proteins had overall amino acid homology of 51%. The two genes had overall nucleotide homology of 64%.

E. coli DH1 harboring plasmid pCBPLC3 also had phospholipase C activity in the osmotic shock fluid, although in this case the activity was 100 to 200 times lower than that of DH1(pPLC8). *E. coli* DH1(pCBPLC3) colonies were only weakly hemolytic on erythrocyte plates.

Characterization of phospholipase C made in *E. coli* DH1(pPLC8) and DH1(pCBPLC3). To compare the phospholipase C made in *E. coli* DH1(pPLC8) and DH1(pCBPLC3) to that of *C. perfringens*, we purified the enzyme from all three sources. Although many procedures have been reported for purification of *C. perfringens* phospholipase C, few have succeeded in isolating the enzyme with high yield without contamination with other toxins. We found the simplest and most efficient method to be the one described by Eloy et al. (7), with some modifications. We purified phospholipase C from *C. perfringens* to more than 95% purity with an overall yield of 0.5 mg per liter of culture.

Similar procedures were used to purify phospholipase C from *E. coli* DH1(pPLC8) and DH1(pCBPLC3). Because of the overexpression of the two cloned genes in *E. coli* and because the enzymes were concentrated in the periplasmic space, purification of phospholipase C from these two sources was simpler than purification of the enzyme from *C. perfringens*. The overall yield was 10 mg per liter for DH1(pPLC8) and 2 mg per liter for DH1(pCBPLC3). The purity of the enzyme from all three sources is shown in Fig. 4.

Both enzymatic and hemolytic specific activities of phospholipase C derived from *E. coli* DH1(pPLC8) were similar to those of the *C. perfringens* enzyme (Table 1). Based on its activities, periplasmic location, and molecular weight as measured by SDS-polyacrylamide gel electrophoresis, this enzyme was probably processed and secreted correctly in *E. coli*. The high yield of this protein from *E. coli* DH1(pPLC8) also indicated that the *C. perfringens* phospholipase C gene was efficiently expressed in *E. coli* despite the phylogenetic difference between the two bacteria.

C. bifermentans phospholipase C isolated from DH1 (pCBPLC3) had very low enzymatic and hemolytic activities compared with the *C. perfringens* enzyme, about 50-fold in each case (Table 1). This protein also had a slightly slower electrophoretic mobility on SDS-polyacrylamide gels than did the *C. perfringens* phospholipase C (Fig. 4). The apparently higher molecular weight protein was further purified from other contaminating proteins by nondenaturing polyacrylamide gel electrophoresis and was shown to have phospholipase C activity (data not shown). It is possible that the signal peptide was not removed from the enzyme in DH1(pCBPLC3). However, replacing the signal peptide sequence in pCBPLC3 with that of the *C. perfringens* phospholipase C gene also resulted in the production of a protein similar in electrophoretic mobility to the isolated *C. bifermentans* enzyme from DH1(pCBPLC3) (data not

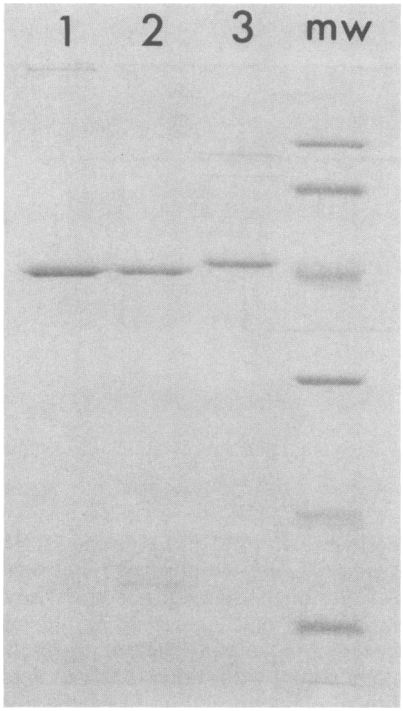

FIG. 4. SDS-polyacrylamide gel electrophoresis of phospholipase C isolated from *C. perfringens* (lane 1), *E. coli* DH1(pPLC8) (lane 2), and *E. coli* DH1(pCBPLC3) (lane 3). Electrophoresis on a 12.5% polyacrylamide gel was performed according to the procedure of Laemmli (14). The gel was stained with Coomassie blue for proteins. Molecular weight (mw) standards: phosphorylase *b*, 94,000; bovine serum albumin, 67,000; ovalbumin, 43,000; carbonic anhydrase, 30,000; soybean trypsin inhibitor 20,000; α-lactalbumin, 14,400.

shown). It is therefore more likely that the anomaly in electrophoretic mobility of this protein was due to its amino acid composition.

Miles and Miles (16) reported that at similar levels of enzyme activity, the *C. bifermentans* phospholipase C is less toxic to animals than is the *C. perfringens* protein. However, these experiments were performed with very crude enzyme preparations and possibly contained materials that might interfere with enzymatic assays and toxicity tests. To verify their observations, we used the purified *C. bifermentans* phospholipase C isolated from the osmotic shock fluid of *E. coli* DH1(pCBPLC3) for toxicity tests in animals. As controls, we used phospholipase C from *C. perfringens* and *E. coli* DH1(pPLC8). Preparations from all three sources were injected intravenously into 8-week-old BALB/c mice and scored for lethality after 24 h. Phospholipase C from either

TABLE 1. Enzymatic and hemolytic activities of phospholipase C from *C. perfringens*, *E. coli* DH1(pPLC8), and *E. coli* DH1(pCBPLC3)

Phospholipase C from:	Sp act	
	Enzymatic (U/mg)	Hemolytic (HU_{50}/mg)[a]
C. perfringens	300	700,000
E. coli DH1(pPLC8)	252	520,000
E. coli DH1(pCBPLC3)	5	12,000

[a] HU_{50}, 50% hemolytic units.

TABLE 2. Toxicity of phospholipase C from *C. perfringens*, *E. coli* DH1(pPLC8), and *E. coli* DH1(pCBPLC3)

Phospholipase C from:	Dose (ng)	Enzymatic activity (U)	No. of mice[a] died/group
C. perfringens	30	9×10^{-3}	0/3
	100	3×10^{-2}	3/3
	300	9×10^{-2}	3/3
E. coli DH1(pPLC8)	100	2.5×10^{-2}	2/3
	300	7.5×10^{-2}	3/3
E. coli DH1(pCBPLC3)	1,000	3×10^{-3}	0/2
	5,000	1.5×10^{-2}	2/2

[a] Eight-week-old BALB/c mice.

C. perfringens or *E. coli* DH1(pPLC8) was very toxic to the animals, having a 50% lethal dose in the range of 30 to 100 ng per animal (Table 2), or 1.5 to 5 μg per kg of body weight, in agreement with the published data for this enzyme (3, 21). At a similar level of enzymatic activity, the *C. bifermentans* phospholipase C was also toxic to animals, in contradiction of the observation of Miles and Miles. It appeared in all three cases that toxicity to animals correlated with the enzymatic activity of the protein.

DISCUSSION

In this paper, we report the cloning and sequencing of two clostridial phospholipase C genes. The approach we took to cloning the *C. perfringens* gene was first to shotgun the restriction fragments of the chromosomal DNA into an *E. coli* plasmid vector and then to screen *E. coli* transformants harboring these plasmids for production of hemolysins. *C. perfringens* cells produce two well-characterized hemolysins, phospholipase C and θ-toxin. The latter is a thio-activated cytolysin (17, 22) and is one of the major proteins secreted by *C. perfringens*. Only the phospholipase C gene was isolated by our cloning method. θ-Toxin, on the other hand, did not show up on our screen. The reason for its absence is not clear. It is possible that the θ-toxin gene was not effectively expressed in *E. coli* or that it was toxic to the cells when expressed. Surprisingly, overexpression of phospholipase C in *E. coli* seemed to have little effect on the cells despite its membrane-damaging action. Bacterial membrane differs from mammalian cell membrane in many ways: the absence of sterols, little or no phosphatidylcholine, and large amounts of odd-numbered branch-chain fatty acids. These compositional differences probably render the *E. coli* cell membrane resistant to phospholipase C in the same way that *C. perfringens* cells are not affected.

We searched the NBRF Protein Identification Resource for proteins with sequence homology to the deduced amino acid sequence of phospholipase C. No sequences with significant homology were found. However, we did find sequence homology between the *C. perfringens* phospholipase C gene and the recently sequenced *Bacillus cereus* phospholipase C gene (12). The *B. cereus* enzyme (molecular weight, 27,000) is nonhemolytic and nontoxic to animals. Although the overall sequence identity is only 28% between the two proteins, there are three blocks of strong homology that indicate relatedness. The most significant block is located between Lys-143–Ile-192 of the *C. perfringens* enzyme and Lys-145–Val-196 of the *B. cereus* protein, where the sequence identity is 46%. The other two, shorter blocks are located between His-96–Asp-101 and His-107–Asp-112 (80% identity) and Gln-257–Ile-264 and Gln-266–Ile-273 (75% identity) of the *C. perfringens* and the *B. cereus* protein,

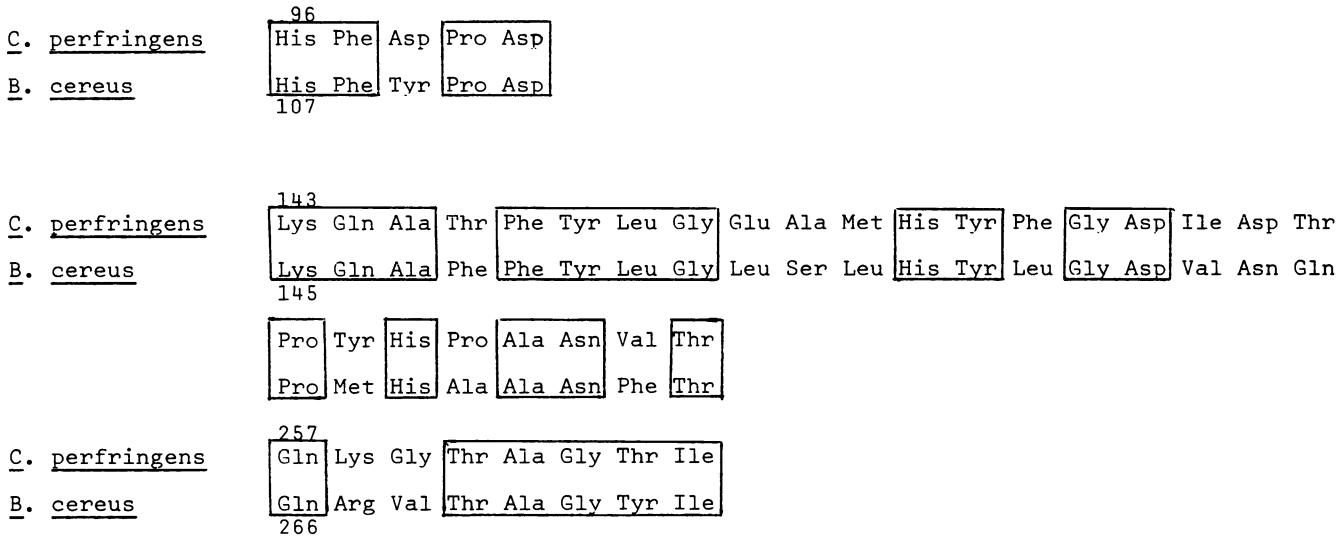

FIG. 5. Comparisons of the primary structures of phospholipase C from *C. perfringens* and *B. cereus*. Three significant blocks of sequence homology are shown. Amino acid residues are numbered from the translation initiation Met residue.

respectively (Fig. 5). This sequence alignment suggests that the phospholipase C catalytic domain is located at the NH_2-terminal portion of the *C. perfringens* protein, while the COOH-terminal unmatched sequence performs additional functions. Between the *C. perfringens* and *C. bifermentans* enzymes, the overall sequence homology is 51%. However, the sequences are significantly more homologous in the NH_2-terminal half of the protein than in the COOH-terminal half, with 60% identity in the former versus 45% in the latter. The only other bacterial phospholipase C gene cloned and sequenced to date is from *Pseudomonas aeruginosa* (19). The mature *P. aeruginosa* phospholipase C (molecular weight, 78,199) has biological activities similar to those of the *C. perfringens* enzyme, although it is about 60- to 140-fold less toxic to animals (2). No significant sequence homology was found between the two proteins.

Cloning of the *C. perfringens* phospholipase C gene provides an improved source of this enzyme for structure-function studies. *E. coli* DH1 harboring this gene gave a 20-fold-higher yield of phospholipase C than did *C. perfringens*. The protein obtained is also totally devoid of other clostridial toxins and hydrolytic enzymes; therefore, the effect of phospholipase C on the cell membrane or in animals as a toxin can be studied more directly. Results in this report have demonstrated that phospholipase C alone can cause death to animals when injected intravenously. Furthermore, having the cloned gene will allow us to do experiments such as in vitro mutagenesis to study the structure-function relationships in this protein. For example, it will be of interest to determine whether it is possible to separate the catalytic activity from the hemolytic and lethal activities by mutations.

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