Arcanobacterium haemolyticum Phospholipase D Is Genetically and Functionally Similar to Corynebacterium pseudotuberculosis Phospholipase D†

WILLIAM A. CUEVAS† AND J. GLENN SONGER*

Department of Veterinary Science, University of Arizona, Tucson, Arizona 85721

Received 18 June 1993/Returned for modification 14 July 1993/Accepted 27 July 1993

Arcanobacterium haemolyticum, a pathogen of the human upper respiratory tract and other systems, has been reported to produce soluble toxins, including a phospholipase D (PLD). We confirmed production of PLD by this organism and cloned and sequenced pld. Arcanobacterial PLD (PLD-A) was found to be a protein of \( \approx 31.5 \) kDa with a pl of \( \approx 9.4 \). Cosmid cloning, followed by subcloning into phagemid pBluescriptIISK+, yielded Escherichia coli(pAh140), a recombinant with a gene product corresponding to PLD-A. Evidence of PLD activity was found by three assays in supernatant fluid of cultures of E. coli(pAh140) and A. haemolyticum, but not in E. coli(pBluescriptIISK+). Experiments to determine if this protein was secreted were not conducted, but previous work with PLD from Corynebacterium pseudotuberculosis suggested that the presence of the enzyme in culture supernatant fluids was due to lysis of E. coli rather than to active transport. Antibodies in polyclonal sera from goats immunized with native or recombinant PLD-A neutralized native and recombinant PLD-A activity, and antibodies associated with native or recombinant PLD from C. pseudotuberculosis (PLD-P) partially neutralized native and recombinant PLD-A. Antibodies prepared against recombinant PLD-A labelled both recombinant and native PLD-A in Western blots (immunoblots) and dot blots, but antibodies against PLD-P did not. Sequencing of the insert in pAh140 revealed an open reading frame of 930 bp coding for 309 amino acids, including a putative signal sequence of 26 amino acids (3.2 kDa, determined on the basis of homology with the 24-amino-acid signal sequence of pld from C. pseudotuberculosis bv. ovis) and the mature PLD protein (31.5 kDa). Sequence comparisons of coding regions revealed 65% DNA homology with pld genes from C. pseudotuberculosis and Corynebacterium ulcerans. Comparison of amino acid sequences revealed 64% homology of PLD-A both with PLD-P and with PLD produced by C. ulcerans.

Arcanobacterium (Corynebacterium) haemolyticum was originally associated with outbreaks of pharyngitis in humans (21, 22, 24). Evidence supporting a role for this organism as an etiologic agent of pharyngitis includes its isolation as the sole or predominant species in the absence of other recognized bacterial pathogens, its absence from follow-up cultures of recovered patients (3, 24–27, 31), and the presence of high titers of specific antibody in clinical cases (11, 47). Failure of some investigators to exclude viral pathogens has left the primary etiologic role of A. haemolyticum in doubt (30), and it is possible that A. haemolyticum behaves as an opportunistic pathogen.

Pharyngitis and cutaneous infections are the most common clinical syndromes associated with A. haemolyticum infection; most of the 150 strains isolated by MacLean and coworkers (24) came from such infections. Most patients thought to have had A. haemolyticum pharyngitis have been young adults presenting with a sore throat, cervical lymphadenopathy, or skin rash. Pharyngitis has been of variable severity, at times mimicking that produced by Streptococcus pyogenes (24, 31) and Corynebacterium diphtheriae (11, 12, 17). The eryhematosus rash observed in half of involved patients has often been a predominant manifestation of disease. Other signs of systemic toxicity, such as fever and leukocytosis, have usually been mild or absent altogether, although A. haemolyticum infection has been misdiagnosed as staphylococcal toxic shock syndrome (43). Isolates of A. haemolyticum have also been obtained from wound infections (22), chronic skin ulcers (24), and brain abscesses (1, 7, 46) and in cases of vertebral osteomyelitis (6) and bacteremia (4, 7, 16).

Toxin production by A. haemolyticum has been reported but remains a subject of controversy. Neither MacLean and coworkers (24) nor Hermann (13) could demonstrate filterable toxins, but in the 1960s, Czechoslovakian investigators published several reports of toxic substances in culture filtrates of A. haemolyticum (29, 37, 38, 40). A toxin or toxins with dermonecrotic and lethal properties appeared in culture supernatant fluids at the beginning of the exponential phase of growth and remained stable for long periods, including during storage at 4° C for at least 1 month (29). In preparations partially purified by ammonium sulfate precipitation or adsorption onto red blood cells, the Czechoslovakian investigators found activity which dissolved egg yolk, hydrolyzed Tween 20, and released choline from lecithin (the last suggesting phospholipase D [PLD] activity) (38). Soucek and coworkers (40) reported that a substance in culture supernatant fluids inhibited the lytic action of \( \beta \)-toxin of Staphylococcus aureus, and the description of the active substances in culture supernatant fluids was expanded to include three elements. An \( \alpha \) component possessed lecinthinase activity, dermonecrotic activity, and \( \beta \)-hemolysin-inhibiting activity (40); adsorbed to erythrocytes; and dissolved egg yolk (39). A \( \beta \) component lysed erythrocytes (39).
and a γ component possessed lipase activity, possibly corresponding to phospholipase A activity (39).

Phospholipases may be important in the pathogenesis of diseases caused by other bacteria, including Photobacterium (Vibrio) damselfa, Clostridium perfringens, Clostridium novyi type D, Pseudomonas aeruginosa, S. aureus, and coryneform organisms such as Corynebacterium pseudotuberculosis and Corynebacterium ulcerans (5, 10, 18, 22, 23, 28, 35, 36, 41, 42, 44). Results of in vivo studies with Pla mutants strongly suggest a role for PLD in the pathogenesis of C. pseudotuberculosis infections in sheep and goats (reference 15 and unpublished results), and our work with this organism has led to an interest in toxic PLDs in general.

The PLD genes and gene products from C. pseudotuberculosis and C. ulcerans have 80% DNA sequence homology and 87% amino acid sequence homology, and the enzymes are antigenically related, as determined by Western blotting (immunoblotting) of the enzyme from C. ulcerans with serum prepared against PLD from C. pseudotuberculosis (unpublished results). A. haemolyticum is similar to pathogenic corynebacteria in many respects and has been considered, until recently, a member of the genus Corynebacterium (8, 9). Strains of A. haemolyticum producing relatively more of the α toxic component (as noted above, possibly a PLD) were often from patients with the most severe manifestations of the disease (37), suggesting a role for the α component in the pathogenesis of A. haemolyticum infections. As an initial step in determining the role of PLD in A. haemolyticum infections and to add to our knowledge of the structure of genes for bacterial toxic phospholipases, we confirmed PLD production by A. haemolyticum and cloned pld from this organism, expressing the gene in Escherichia coli.

MATERIALS AND METHODS

Assays of PLD activity. PLD activity was detected by screening for synergistic hemolysis (with eukaryotic factors [soluble products of Rhodococcus equi ATCC 35701]) and staphylococcal β-hemolysin inhibition (which are in vitro correlates of PLD activity of C. pseudotuberculosis PLD [36]) and confirmed by measuring enzymatic release of the [14C]choline moiety from labelled sphingomyelin (36).

To determine whether the enzyme was specifically PLD rather than phospholipase C, a modification of a procedure for determination of phospholipid concentrations in serum and plasma was used (45). Forty units (as determined by the radiometric assay) of purified enzyme (see below) from A. haemolyticum was added to 500 μl of color reagent (choline oxidase [2 U/ml] [Sigma Chemical Co., St. Louis, Mo.], peroxidase [5 U/ml] [Sigma], 4-aminoantipyrine [0.015%] [Sigma], and phenol [0.05%] in 0.5 M Tris-HCl [pH 8.0]). Portions (10 μl) of sphingomyelin suspension (1 mg/ml in 100 mM Tris-HCl [pH 9.2] with 25 mM NaCl and 5 mM MgCl2) were added; mixtures were vortexed for 15 s and incubated at 37°C for 10 min; and the optical densities at 505 nm were determined using color reagent as a blank and purified PLD-P (10) and Clostridium perfringens α toxin (Sigma) as controls. All assays were performed in duplicate.

In this assay, PLDs produce measurable color change above background values, while phospholipases C produce no color change.

Purification of PLD from A. haemolyticum. PLD from A. haemolyticum ATCC 9345 (PLD-A) was purified to apparent homogeneity for comparison with the recombinant gene product. Briefly, culture supernatant fluids (from 500 ml of brain heart infusion broth culture in 1-liter Erlenmeyer flasks, incubated for 48 h at 37°C, with shaking at 250 rpm) were dialyzed against 20 liters of high-pressure liquid chromatography-grade water, lyophilized, and dissolved in 1 ml of 1 M urea–10% ethylene glycol–150 mM NaCl–200 mM Tris HCl (pH 7.0). This material was fractionated on a Pharmacia C 26/100 column packed with Bio-Gel P-100 (Bio-Rad Laboratories, Richmond, Calif.), eluting with the same buffer at 40 ml/h in 5-ml fractions. Fractions with the highest synergistic hemolytic and β-toxin-inhibiting activities were pooled and dialyzed completely against water. Ammonium sulfate, sodium chloride, and disodium phosphate were added to final concentrations of 0.75 M, 77 mM, and 57 mM, respectively, and the pH was adjusted to 7.0. This material was applied to a Pharmacia C 10/40 column packed with phenyl Sepharose CL-4B (Pharmacia-LKB Biotechnology, Alameda, Calif.) which had been equilibrated with 0.75 M (NH4)2SO4–77 mM NaCl–67 mM Na2HPO4 (pH 7.0). Elution was isocratic at 10 ml/h, in 5-ml fractions.

Fractions with synergistic hemolytic and β-toxin-inhibiting activities were pooled, concentrated 20-fold by ultrafiltration (Amicon stir-cell and YM10 membrane; Amicon, Danvers, Mass.), and dialyzed against 0.9 M NaCl–10% glycerol–0.1 M Tris HCl (pH 7.5). Portions (7 ml) were fractionated on a Pharmacia C 16/100 column packed with Bio-Gel P-60 (Bio-Rad), eluting with the same buffer at 20 ml/h in 5-ml fractions. Fractions were examined by using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (19) and synergistic hemolytic and β-toxin inhibition assays. The isoelectric point was determined by using a gradient of pH 3 to 10 in a Rotofer isoelectric focusing cell (Bio-Rad) (10).

Preparation of arcanobacterial DNA. The washed cell pellet harvested from a 500-ml culture of A. haemolyticum in brain heart infusion broth was suspended in 2.5 mM Tris–2.5 mM EDTA–0.5 M sucrose (pH 8.0) with 10 mg of lysozyme (Sigma) per ml. After incubation at 37°C for 4 h, the protoplasts were harvested by centrifugation and the pellet was suspended in 5 mM Tris–5 mM EDTA–1% SDS (pH 7.4) and incubated at 50°C for 30 min. Cell debris and protein were removed by repeated extraction with 100 mM Tris-saturated phenol (pH 8.0), phenol-chloroform-isoamyl alcohol, and chloroform-isoamyl alcohol. After precipitation with ethanol and drying, the DNA was dissolved in 7 M guanidine hydrochloride (Bethesda Research Laboratories, Gaithersburg, Md.) in Tris-EDTA (pH 8.0) and the mixture was incubated at 55°C for 2 h. The DNA was then precipitated twice with ethanol, the second time in the presence of 0.1 volume of 3 M sodium acetate (pH 5.0).

DNA was prepared by the same method from C. ulcerans, from isolates of biovars equi and ovis of C. pseudotuberculosis (35), and from Actinomycetes (Corynebacterium) pyogenes.

Construction of the arcanobacterial cosmID library. Cosmid pLAFR2 was prepared by alkaline lysis (32) of E. coli LEE392 cultured in Luria-Bertani broth containing 15 μg of tetacycline per ml. Cosmid DNA was purified by centrifugation on a cesium chloride gradient (55,000 rpm, 20°C; VTi 65 rotor; Beckman Instruments, Fullerton, Calif.), digested with BamHII (Promega Biotec, Madison, Wis.), dephosphorylated with calf intestinal alkaline phosphatase (Promega), phenol-chloroform-isoamyl alcohol extracted, and ethanol precipitated (32). About 50 μg of arcanobacterial DNA was partially digested with Sau3AI (Promega) to yield fragments 17 to 30 kb in length (34, 36). These fragments were mixed with the prepared cosmid DNA in a final molar ratio of 3:1 and ligated by using T4 DNA ligase (Promega) at 16°C for 16 h. A 10-μl aliquot of the ligation mixture was packaged with
the Packagene system (Promega) according to the manufacturer’s instructions.

The cosmids library was examined for the PLD gene by transducing E. coli LE392 to tetracycline resistance and screening transductants for synergistic hemolytic activity (an in vitro correlate of PLD activity) on Luria-Bertani agar containing 10% equi factors, 5% ovine erythrocytes, and 15 μg of tetracycline per ml. Plates were incubated at 37°C, with hemolytic colonies appearing after 16 to 20 h. Synergistic hemolytic activity of the gene product was confirmed by streaking these recombinants onto blood agar, alone and in the presence of equi factors. From the several synergistically hemolytic, putatively PLD* recombinants, we selected one clone for further characterization, designating it pAh01.

Agarose gel electrophoretic analysis revealed an ~30-kb insert in pAh01. After complete EcoRI digestion, fragments of the insert were subcloned into the EcoRI site of pBlueScriptIIISK+ and transformed into competent E. coli DH5α. Cells were plated on Luria-Bertani agar with 10% equi factors, 5% ovine erythrocytes, and 100 μg of ampicillin per ml. Hemolytic colonies appeared after 16 to 20 h of incubation at 37°C. The plasmids in these hemolytic recombinants contained a 2.6-kb insert fragment, which was digested with HindIII, ligated into the HindIII site of pBlueScriptIIISK+, and transformed into competent E. coli DH5α. Transformants with a synergistically hemolytic gene product contained a 1.4-kb insert in pBlueScriptIIISK+ and were designated pAh140.

Southern blotting. Chromosomal DNA from A. haemolyticum was digested with HindIII. Aliquots of chromosomal DNA from C. ulcerans, C. pseudotuberculosis, ovis, and Actinomyces pyogenes were digested with HindII. DNA was electrophoresed in 1.0% agarose and transferred to GeneScreen Plus membrane (DuPont-New England Nuclear, Wilmington, Del.) (32). A gene-specific probe was constructed on the basis of sequence data of pAh140 (see below). Flanking sequences of pld were removed by using a combination of subcloning and exonuclease III digestion, yielding a fragment internal to pld. The probe was purified from a large, labelled HindIII digest of pAh140 by the random primer method (Pharmacia-LKB), and hybridized to the membrane at 42°C for 18 h in hybridization solution consisting of 50% formamide, 50 mM K2HPO4, 2× Denhardt’s solution, 0.25 mg of bakers’ yeast tRNA (Sigma) per ml, 0.2% SDS, and 5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate). The membrane was washed at room temperature in 2× SSC-0.1% SDS for 5 min and again for 15 min, in 0.2× SSC-0.1% SDS for 15 min, in 0.1× SSC-0.1% SDS for 15 min, and in 0.1× SSC-1.0% SDS for 30 min, at 50°C. Probing was also carried out under conditions of lower stringency by lowering the hybridization temperature to 32°C and the final wash temperature to 37°C. Autoradiography against Kodak X-Omat AR film was at ~85°C for 16 h.

DNA sequencing. Nested deletions in both DNA strands of pAh140, which contained fragments separated by about 200 to 300 bp, were prepared (2). Ordered deletions were sequenced by the dyeoxy chain termination method (2) by using Sequetide (DuPont/New England Nuclear) according to the manufacturer’s instructions.

Anti-PLD-A serum. Goats were inoculated intravenously with filtered culture supernatant fluids from A. haemolyticum and E. coli(pAh140) administered in 5-ml aliquots weekly for 8 weeks. Serum samples collected from the two goats contained antibodies which neutralized the activity of both native and recombinant PLDs, as measured by the radiometric assay and by inhibition of synergistic hemolysis.

Antibodies against native and recombinant PLD-P were prepared in the same manner.

Electrophoretic examination of native and recombinant proteins. Proteins from cells and/or culture supernatant fluids of A. haemolyticum, E. coli(pBluescriptIISK+), and E. coli(pAh140) were examined by SDS-PAGE. About 50 μg of protein was loaded into each lane, and the gels were run for 4 to 6 h at 25 mA. Gels were fixed overnight in 50% ethanol and 10% glacial acetic acid and then color silver stained by a Gelcode method (Pierce Chemical, Rockford, Ill.) (33).

Proteins in some unstained gels were electrotransferred to nitrocellulose membranes (Bio-Rad) and probed with immune goat serum diluted 1:250 followed by a rabbit anti-goat immunoglobulin G-peroxidase conjugate (Kirkegaard & Perry, Gaithersburg, Md.) diluted 1:1,000. Blots were incubated with peroxidase substrate (Bio-Rad) at room temperature for 5 min (36) following biotin-streptavidin amplification (BLAST System; New England Nuclear).

Neutralizing antibody assay. Aliquots (45 μl) of culture supernatants containing native and recombinant PLD-P and PLD-A were mixed with 5-μl aliquots of sera taken from goats immunized with culture supernatants containing either PLD-A or PLD-P. Sera from nonimmunized goats were used as controls. After incubation at 37°C for 1 h, 10-μl portions of each mixture were placed into wells cut into immunodiffusion plates containing blood agar and 10% equi factors, as previously described (36). Plates were incubated at 37°C for 16 h, and zones of synergistic hemolysis were measured. Zone size was decreased in the presence of normal serum, and values resulting from neutralization tests were adjusted accordingly. This effect of normal serum may have been due to interaction of PLD with serum phospholipids. It may also be due to the presence of endogenous neutralizing antibodies, but the phenomenon is seen with serum from goats and sheep from flocks with no history of caseous lymphadenitis and is also seen with serum from normal rabbits. PLD-serum mixtures were also assayed for PLD activity by using the radiometric sphingomyelinase assay previously described.

Results and Discussion

PLD production by A. haemolyticum. Purification methods described yielded PLD-A purified to homogeneity or near homogeneity, as judged by examination of the purified material in SDS-polyacrylamide gels with silver staining (Fig. 1). No step-by-step estimates of efficiency of the purification process were made, but the final material had been purified ~150-fold and had a specific activity of 1,096 U/mg. Use of the colorimetric assay (45) confirmed that this molecule possessed PLD activity.

PLD-A was similar to PLD-P in molecular mass (~31.5 kDa for PLD-A [Fig. 1] and ~31.7 kDa for PLD-P [10, 20]) as determined by SDS-PAGE and from pl (~9.4 for PLD-A data not shown) and ~9.8 for PLD-P [10, 20]). In isoelectric focusing experiments, most of the PLD-A activity focused at pH 9.4. Unlike PLD-P (11), PLD-A did not focus sharply at its apparent isoelectric pH, and we experienced difficulty in separating PLD-A from other arcanobacterial proteins which focused at this pl. Thus, we were unable to use preparative isoelectric focusing for routine purification. The basis for this result is unknown, but it could possibly be due to the occurrence of multiple isoforms of PLD-A.
Antibodies prepared against whole supernatant fluid from *E. coli* (pAh140) labelled many proteins in supernatant fluids from cultures of *E. coli* (pBluescriptIIISK+) and one additional protein in supernatant fluids from cultures of *E. coli* (pAh140) (Fig. 2, lanes 1 and 2). In supernatant fluid from cultures of *A. haemolyticum*, a protein of ≈31.5 kDa, corresponding to the molecular mass of PLD-A, was labelled (Fig. 2, lane 3). The labelling of a group of high-molecular-mass proteins in supernatant fluids from cultures of *A. haemolyticum* is unexplained. Antibodies against purified native PLD-P failed to label PLD-A in Western or dot blots, as did serum from normal goats (data not shown). The failure of antibodies against antigens in culture supernatant fluids of *C. pseudotuberculosis* to recognize antigens of *A. haemolyticum* in gel diffusion assays has also been reported by others (29).

Although *A. haemolyticum* is hemolytic when grown on blood agar, we did not encounter independent hemolytic activity in fractions at any step in the purification process. Screening of several independent libraries, representing ≈50-fold coverage of the genome of *A. haemolyticum*, did not reveal any recombinant producing an independently hemolytic gene product. Thus, we cannot confirm other findings (39, 40) regarding production of a hemolysin by this organism, but these results parallel those from our studies with *C. pseudotuberculosis* (36) and suggest, while not confirming, that *A. haemolyticum* does not produce a hemolysin independent of PLD-A. On the other hand, if an oxygen-labile hemolysin was present, it might have gone undetected by our methods. It may also be that *A. haemolyticum* produces sufficient hemolysin to lyse erythrocytes on a plate but insufficient quantities to be detected in our assay (the β-toxin inhibition assay before addition of staphylococcal β-toxin).

**Cloning and characterization of pld from *A. haemolyticum***

Cosmid cloning, followed by subcloning to phagemid pBluescriptIIISK+, yielded *E. coli* (pAh140), a recombinant with a gene product corresponding to PLD-A. On the basis of estimates of the molecular weight of PLD-A (see above), the 1.4-kb insert in pAh140 was determined to be sufficient to span the structural gene and accessory sequences.

To confirm that pAh140 contained *pld*, PLD activities in *E. coli* (pAh140), *E. coli* (pBluescriptIIISK+), and *A. haemolyticum* ATCC 9345 were compared. First, culture supernatant fluids were examined for synergistic hemolytic, β-toxin-inhibiting, and PLD activities. Supernatant fluids from cultures of *E. coli* (pAh140) and of *A. haemolyticum*, but not from cultures of *E. coli* (pBluescriptIIISK+), exhibited synergistic hemolytic and β-toxin-inhibiting activities, both of which are characteristic of PLD-P (36). Supernatant fluids from cultures of *E. coli* (pAh140) and of *A. haemolyticum*, examined by the radiometric assay for release of labelled choline moiety from [14C]sphingomyelin, contained PLD activity, but no activity was produced by *E. coli* (pBluescriptIIISK+). Detection of PLD activity in supernatants of cultures of *E. coli* (pAh140) does not imply active transport of the enzyme by *E. coli*. Experiments were not done to confirm this, but earlier experience with the PLD gene from *C. pseudotuberculosis*, cloned into *E. coli*, showed that most recombinant PLD activity remained in association with the bacterial cells (36).

Second, purified native PLD and supernatant fluids from cultures of *A. haemolyticum* and of *E. coli* (pAh140) were examined by SDS-PAGE and Western blotting (Fig. 1 and 2). A major product of *E. coli* (pAh140), which was labelled by goat anti-recombinant-PLD-A antibodies, had a molecular size of ≈31.5 kDa and was present in lanes loaded with *E. coli* (pAh140) supernatant fluid but not in lanes loaded with that of *E. coli* (pBluescriptIIISK+). Anti-recombinant-PLD-A antibodies also labelled a ≈31.5-kDa product present in supernatant fluids from cultures of *A. haemolyticum* (Fig. 2). The discrepancy between molecular sizes of recombinant and native PLD-A may be due to a lack of posttranslational modification of recombinant PLD-A, specifically, to a lack of signal sequence cleavage.

Third, the ability of antibodies in polyclonal sera from immunized goats to neutralize PLD-A activity was deter-
TABLE 1. Neutralization of PLDs by homologous and heterologous sera

<table>
<thead>
<tr>
<th>Serum</th>
<th>Native</th>
<th>Recombinant</th>
<th>Native</th>
<th>Recombinant</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-native PLD-P</td>
<td>100</td>
<td>100</td>
<td>49.7</td>
<td>47.4</td>
</tr>
<tr>
<td>Anti-recombinant PLD-P</td>
<td>78.1</td>
<td>100</td>
<td>36.3</td>
<td>16.9</td>
</tr>
<tr>
<td>Anti-native PLD-A</td>
<td>13.4</td>
<td>11.4</td>
<td>100</td>
<td>94.6</td>
</tr>
<tr>
<td>Anti-recombinant PLD-A</td>
<td>87.5</td>
<td>73.8</td>
<td>100</td>
<td>100</td>
</tr>
</tbody>
</table>

* Aliquots (45 μl) of culture supernatants containing native and recombinant PLD-P and PLD-A were mixed with 5-μl aliquots of serum taken from goats immunized with PLD-A or PLD-P. After 1 h of incubation at 37°C, 10 μl of each mixture was assayed for synergistic hemolytic activity (see text). Zone area (in square millimeters) was adjusted on the basis of controls (normal serum).

mined (Table 1). Percent neutralization was calculated by comparison of the areas of the zones of synergistic hemolytic activity produced by mixtures of PLD-A and immune sera prepared against recombinant or native PLD-A and PLD-P. Sera from goats immunized with either native or recombinant PLD-A inhibited the activity of both recombinant and native PLD-A. These results corresponded to those obtained with the radiomicroassay of sphingomyelinase activity (data not shown). Partial neutralization of native and recombinant PLD-A by antisera prepared against native or recombinant PLD-P was unexpected, because of the results of our Western blots and of gel diffusion assays reported by others (29), in which no interaction between antibodies against PLD-P and antigens from PLD-A was observed. The reasons for this are not known but are probably not related to the amount of antigen applied to the filter, since both Western blots (perhaps with smaller amounts of antigen) and dot blots (with substantially more antigen) were negative. Native epitopes which are conserved and which result in PLD activity in neutralization assays may be denatured by SDS and rendered unrecognizable to antibodies in Western blots. It may also be that the neutralizing antibodies produced here are not active in Western blots, for unknown reasons. These results remain anomalous, and clarification will require further experimental work.

On the basis of (i) production of PLD by *E. coli* (pAh140), (ii) neutralization of native and recombinant PLD by goat anti-PLD sera, (iii) antibody labelling of native and recombinant proteins of similar molecular mass, and (iv) lack of evidence for other synergistically hemolytic or sphingomyelin-hydrolyzing proteins in *A. haemolyticum*, the ~31.5-kDa protein produced by *E. coli* (pAh140) must be PLD-A and pld must be contained in pAh140.

Comparison of the restriction maps of *pld* from *A. haemolyticum* (not shown) and from *C. pseudotuberculosis* (36) revealed no similarities sufficient to indicate the position of the gene within the cloned fragment. Sequencing of nested deletions of pAh140 revealed an open reading frame of 930 bp coding for 309 amino acids, including a putative signal sequence of 26 amino acids (3.2 kDa), determined on the basis of homology with the 24-amino-acid signal sequence of *pld* from *C. pseudotuberculosis* (20 kDa) (14) and the mature PLD protein (31.5 kDa). Thus, the *pld* protein from *C. pseudotuberculosis* and the *pld* protein from *A. haemolyticum* are each apparently composed of 283 amino acid residues (14). Confirmation of these observations would require N-terminal amino acid sequencing, which was not done. Regions with partial homology to *E. coli* ~35 and ~10 promoter regions were also found (Fig. 3). Sequence comparison of *pld* genes from *A. haemolyticum* with those from *C. pseudotuberculosis* (reference 14 and unpublished results) and *C. ulcerans* (our unpublished results) revealed 65% DNA homology within the coding region. Comparison of amino acid sequences revealed 64% homology of PLD-A with both PLD-P and PLD produced by *C. ulcerans*. A DNA homology search of GenBank revealed no significant similarities between arcanobacterial or corynebacterial *pld* genes and other phospholipase or toxin genes.

A DNA probe constructed from a purified internal *pld* sequence derived from pAh140 was used in Southern blots with target DNA consisting of restriction enzyme-digested chromosomal DNA from *A. haemolyticum*, *C. ulcerans*, *C. pseudotuberculosis* bv. equi and ovis, and *Actinomycetes pyogenes*. Surprisingly, a single band of 3.2 kb was observed in lanes containing *A. haemolyticum* chromosomal DNA cut
with HindIII (data not shown). The inconsistency between the size of the HindIII subcloned insert used as a probe and the size of the HindIII-digested chromosomal band with which it hybridized remains unexplained but may be due to the presence of restriction sites in arcanobacterial DNA which are cleaved only when prepared in *E. coli*. This may be due to the presence of a DNA modification system in *A. haemolyticum* which is absent in *E. coli* DH5α.

Under conditions of high stringency, Southern blot analysis demonstrated no homology between the internal pld fragment and chromosomal DNA from *C. ulcerans*, *C. pseudotuberculosis*, and *Actinomyces pyogenes*. However, at lower stringency (80 to 85%), a single band of 1.8 kb was observed in lanes containing HindII-digested chromosomal DNA from *C. pseudotuberculosis* bv. equi and ovis. This result was as predicted, in that the genes coding for PLD in the two *C. pseudotuberculosis* biovars were found on 1.8-kb HindII DNA fragments (reference 36 and unpublished results).

The results are useful in that they confirm the production of PLD by *A. haemolyticum* and reveal similarities between PLD-A, PLD-P, and PLD produced by *C. ulcerans*. Lack of significant DNA homology between these pld genes and sequences in GenBank may imply unique structural features within this group of enzymes. Comparisons with sequences of eukaryotic PLDs, when these become available, may be of interest. Further study of the genes, by site-directed mutagenesis and other methods, may reveal aspects of structure and function which will contribute to our understanding of the various in vitro correlates of PLD activity (e.g., whether PLD, synergistic hemolysis, and β-hemolysin inhibition are one and the same or arise from two or more different molecular domains) and perhaps suggest a role of these enzymes in pathogenesis of infections by the respective organisms.

ACKNOWLEDGMENTS

This work was supported in part by funds from the U.S. Department of Agriculture (project numbers 87-CSRSA-2-3118, 88-CR8R-34116-3750, 91-37204-6683, and ARZT-174861-H-02-06) and by BRSG S07 RR07002, awarded by the Biomedical Research Support Grant Program, Division of Research Resources, National Institutes of Health.

REFERENCES

7. Chandraeskar, P. H., and J. A. Molinari. 1987. *Corynebacterium haemolyticum* bacteremia with fatal neurologic complica-
13. Hermann, G. J. 1961. The laboratory recognition of *Corynebac-
20. Linder, R., and A. W. Bernheimer. 1978. Effect on sphingomyelin-containing liposomes of phospholipase D from *Corynebac-
23. Low, D. K. R., and J. H. Freer. 1977. Biological effects of highly purified β-lysin (sphingomyelinase C) from *Staphylococcus aure-
teria haemolyticum* as a cause of pharyngitis and scarlati-