

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

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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 ≈31.5 kDa with a pI of ≈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 against 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 erythematous 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 β-toxin of *Staphylococcus aureus*, and the description of the active substances in culture supernatant fluids was expanded to include three elements. An α component possessed lecithinase activity, dermonecrotic activity, and β-hemolysin-inhibiting activity (40); adsorbed to erythrocytes; and dissolved egg yolk (39). A β component lysed erythrocytes (39),

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and a τ 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*) *damisela*, *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 PLD⁻ 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 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 equi factors [soluble products of *Rhodococcus equi* ATCC 33701]) and staphylococcal β -hemolysin inhibition (which are in vitro correlates of PLD activity of *C. pseudotuberculosis* [PLD-P] [10, 35, 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 μ l of color reagent (choline oxidase [2 U/ml] [Sigma Chemical Co., St. Louis, Mo.], peroxidase [5 U/ml] [Sigma], 4-aminoantipyrene [0.015%] [Sigma], and phenol [0.05%] in 0.05 M Tris-HCl [pH 8.0]). Portions (10 μ l) of sphingomyelin suspension (1 mg/ml in 100 mM Tris-HCl [pH 9.2] with 25 mM NaCl and 5 mM MgCl₂) were added; the reaction mixtures were vortexed for 15 s and incubated at 37°C for 10 min; and the optical densities at 505 nm were determined by 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 10 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 67 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 Rotofor 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 *Actinomyces* (*Corynebacterium*) *pyogenes*.

Construction of the arcanobacterial cosmid library. Cosmid pLAFR2 was prepared by alkaline lysis (32) of *E. coli* LE392 cultivated in Luria-Bertani broth containing 15 μ g of tetracycline 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 *Bam*HI (Promega Biotec, Madison, Wis.), dephosphorylated with calf intestinal alkaline phosphatase (Promega), phenol-chloroform-isoamyl alcohol extracted, and ethanol precipitated (32). About 50 μ g of arcanobacterial DNA was partially digested with *Sau*3A (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- μ l aliquot of the ligation mixture was packaged with

the Packagene system (Promega) according to the manufacturer's instructions.

The cosmid 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 pBlue-scriptIISK⁺ 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 pBlue-scriptIISK⁺, and transformed into competent *E. coli* DH5α. Transformants with a synergistically hemolytic gene product contained a 1.4-kb insert in pBlue-scriptIISK⁺ and were designated pAh140.

Southern blotting. Chromosomal DNA from *A. haemolyticum* was digested with *HindIII*. Aliquots of chromosomal DNA from *C. ulcerans*, *C. pseudotuberculosis* bv. equi and ovis, and *Actinomyces pyogenes* were digested with *HincII*. 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 an agarose gel, labelled with ³²P 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 dideoxy chain termination method (2) by using ³⁵S-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*(pBlue-scriptIISK⁺), 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.

Nucleotide sequence accession number. The sequence of *pld* from *A. haemolyticum* has been assigned GenBank accession number L16583.

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 pI (≈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 pI. 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.

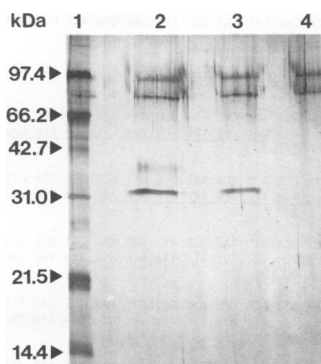


FIG. 1. Silver-stained, SDS-12.5% polyacrylamide gel containing fractions from size exclusion column P60 (final native PLD-A purification step). Lane 1, molecular mass standards; lane 2, fraction 4, exhibiting partial purification; lane 3, fraction 5, the purest fraction obtained; lane 4, running and loading buffers, containing artifactual bands.

Antibodies prepared against whole supernatant fluid from *E. coli*(pAh140) labelled many proteins in supernatant fluids from cultures of *E. coli*(pBluescriptIISK+) 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. haemolyti-*

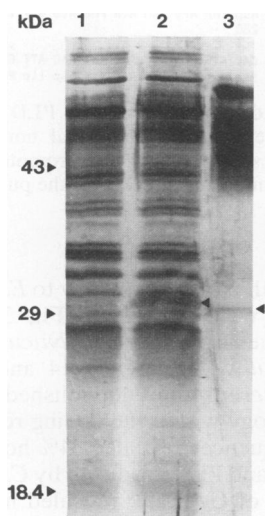


FIG. 2. Western blot analysis of native and recombinant PLD-A. Proteins were transferred from an SDS-12.5% polyacrylamide gel to nitrocellulose membrane and probed with serum from a goat immunized with supernatant fluid from a culture of *E. coli*(pAh140). Lanes 1 through 3, supernatant fluids from cultures of *E. coli*(pBluescriptIISK+), *E. coli*(pAh140), and *A. haemolyticum*, respectively. Arrowheads adjacent to lane 1 indicate molecular masses, and those adjacent to lanes 2 and 3 indicate the positions of recombinant PLD-A and of native PLD-A, respectively.

cum 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 into phagemid pBluescriptIISK+, 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*(pBluescriptIISK+), 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*(pBluescriptIISK+), 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 [14 C]sphingomyelin, contained PLD activity, but no activity was produced by *E. coli*(pBluescriptIISK+). 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*(pBluescriptIISK+) (Fig. 2). Other protein bands were detected but were identical in molecular size to labelled bands present in lanes loaded with supernatant fluid from *E. coli*(pBluescriptIISK+). 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^a

Serum	% Reduction in zone area with:			
	PLD-P		PLD-A	
	Native	Recombinant	Native	Recombinant
Anti-native PLD-P	100	100	49.7	47.4
Anti-recombinant PLD-P	78.1	100	36.3	16.9
Anti-native PLD-A	13.4	11.4	100	94.6
Anti-recombinant PLD-A	87.5	73.8	100	100

^a Aliquots (45 μ l) of culture supernatants containing native and recombinant PLD-P and PLD-A were mixed with 5- μ l aliquots of serum taken from goats immunized with PLD-A or PLD-P. After 1 h of incubation at 37°C, 10 μ l 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 hemolysis 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 radiometric assay of sphingomyelinase activity (data not shown). Partial neutralization of native and recombinant PLD-A by antiserum 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 \approx 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* bv. ovis) (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

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1 GCA AAG TGT AAC GGT CAA CAT AGA GAC ATG GAA GAG AAC ACC ACA GGT TCT GAA AAA CAT
(-35)
61 TGT GAA TAT TTC TCT TTC ATC ATA TAA ATA CAC AAT TTT ATC CTT AAC CGG ATG GGC TTC
(-10)
121 GCA GAA AGA TGT ACG ACG ATG AAG ACG CGG AAA AAA ATT GCT TTA GCC TTG TCG CTT CTC
Met Lys Thr Arg Lys Lys Ile Ala Leu Ala Leu Ser Leu Leu
10
181 ACG GGA TTT ATG CTA CCA ATT GGT AGT GCG GCT GCT GCG CCA CTT GCG CAA GAA CAA CCA
Thr Gly Phe Met Leu Pro Ile Gly Ser Ala Ala Ala Ala Pro Leu Leu Ala Gln Glu Gln Pro
20
241 ACC ACT GGT AAC CGT CCA GTC TAT GCC ATT GCG CAC GGT GAT CTG ACG AAG CAA AGT GTC
Thr Thr Gly Asn Arg Pro Val Tyr Ala Ile Ala His Arg Val Leu Thr Lys Gln Ser Val
40
301 GAT GAC GCA ATC AAA ATT GGC GCG AAC GCT CTG GAA ATT GAT TTT ACT GCG TGG CGC CGT
Asp Asp Ala Ile Lys Ile Gly Ala Asn Ala Leu Glu Ile Asp Phe Thr Ala Trp Arg Arg
60
361 GGC TGG TGG GCT GAC CAT GAC GGG CTT CCA ACT AGT GCT GGC GAT ACG GCA GAA GAC ATT
Gly Trp Trp Ala Asp His Asp Gly Leu Pro Thr Ser Ala Gly Asp Thr Ala Glu Asp Ile
80
421 CTT AAG TAC ATC GCT CAA AAG CGT CGC GAA GGT AAT AAC ATT ACA TTT GTC TGG TTT GAT
Leu Lys Tyr Ile Ala Gln Lys Arg Arg Glu Gly Asn Asn Ile Thr Phe Val Trp Phe Asp
100
481 ATT AAG AAC CCA GAT TAT TGT AAG GAC CAA AAT AGT GTG TGT TCA ATT ACG AAG CTA AGA
Ile Lys Asn Pro Asp Tyr Cys Lys Asp Gln Asn Ser Val Cys Ser Ile Thr Lys Leu Arg
120
541 GAT CTC GCA CGG CAA ACG ATT GAA CAA GAA GGC GTC AGA GCT CTC TTT GGC TAT TAT AAG
Asp Leu Ala Arg Gln Thr Ile Glu Gln Glu Gly Val Arg Ala Leu Phe Gly Phe Tyr Lys
140
601 ACA GTC GGA GGT GTC GGC TGG AAT ACT ATT GCT AAT AAT CTC AAC GAC AAG GAA GCA GTT
Thr Val Gly Gly Val Gly Trp Asn Thr Ile Ala Asn Asn Leu Asn Asp Lys Glu Ala Val
160
661 GCT CTC AGT GGC CGA AAA GAT GAC ATC ATG AAG GAC TTC AAG CAG TAC GAA AAT AAG ATC
Ala Leu Ser Gly Arg Lys Asp Asp Ile Met Lys Asp Phe Lys Gln Tyr Glu Asn Lys Ile
180
721 AAA CCT CAG CAA CGC GTC GCT GAT AAC GGT TAC TAC AAC CTT AGC TAC GGC TTT GGT GGT
Lys Pro Gln Gln Arg Val Ala Asp Asn Gly Tyr Tyr Asn Leu Ser Tyr Gly Phe Gly Gly
200
781 TGC TAC CGA GAT GAG AAT CAG ACG TGT GAT CAG CTG CGT CTT GCT GGA GAA GAA CGC AAA
Cys Tyr Arg Asp Glu Asn Gln Thr Cys Asp Gln Leu Arg Leu Ala Gly Glu Glu Arg Lys
220
841 AAG GGG AAT CTA GGA AAG ACC TTC GGA TGG ACC GTC AGT ACT GGA CAG GAA TAC CTG GCT
Lys Gly Asn Leu Gly Lys Thr Phe Gly Trp Thr Val Ser Thr Gly Gln Glu Tyr Leu Ala
240
901 GCA GAT CTT CTG AAT AAG GCA GAA GTT GAT GGA ATG ATC TTC GGA TTT AAG ACC ACC TAC
Ala Asp Leu Leu Asn Lys Ala Glu Val Asp Gly Met Ile Phe Gly Phe Lys Thr Thr Tyr
260
961 TTC TAT GAT CAC GCC GAT ACT CGC AAT GCT TTT GCT GGA ATT AAG AAC TGG GTG GAT GCA
Phe Tyr Asp His Ala Asp Thr Arg Asn Ala Phe Ala Gly Ile Lys Asn Trp Val Asp Ala
280
1021 CAT CAA GGT ACT CAC CAC ATG GCA ACC AAC AAA GAC ATT CCG TGG TGA TCG AAT GAG ATA
His Gln Gly Thr His His Met Ala Thr Asn Lys Asp Ile Pro Trp End

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FIG. 3. Nucleotide sequence of the PLD gene from *A. haemolyticum*. Base sequences with partial homology with *E. coli* consensus sequences for -10 and -35 promoter regions are underlined. Underlined amino acids represent the putative signal peptide.

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 *Actinomyces pyogenes*. Surprisingly, a single band of 3.2 kb was observed in lanes containing *A. haemolyticum* chromosomal DNA cut

with *Hind*III (data not shown). The inconsistency between the size of the *Hind*III subcloned insert used as a probe and the size of the *Hind*III-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* DH5 α .

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 *Hinc*II-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 *Hinc*II 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.

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