INFECTION AND IMMUNITY, Jan. 1990, p. 131–136
0019-9567/90/010131-06$02.00/0

Vol. 58, No. 1

Cloning and Expression of the Phospholipase D Gene from *Corynebacterium pseudotuberculosis* in *Escherichia coli*†

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Received 16 May 1989/Accepted 6 October 1989

A toxic phospholipase D (PLD) is putatively involved in pathogenesis of *Corynebacterium pseudotuberculosis* infections. We report here the cloning and expression of the PLD gene (*pld*) in *Escherichia coli*. A cosmid library of DNA from *C. pseudotuberculosis* biovar *ovis* isolate Whetten 1 was constructed and screened for PLD-producing recombinants by plating them on LB agar containing sheep erythrocytes and *equi* factors. One recombinant, designated pCpO1, yielded a gene product which displayed synergistic hemolytic and sphingomyelinase D activities, both of which are characteristic of PLD. Subcloning into pUC19 yielded a recombinant, pCpO50, which contained a 1.8-kilobase insert. Analysis of supernatant fluids and cell extracts of cultures of *E. coli*(pCpO50) revealed sphingomyelinase activity and a protein of about 31,000 M_r, neither of which were detected in *E. coli*(pUC19). The 31-kilodalton protein also reacted with antibodies in serum from a sheep naturally infected with *C. pseudotuberculosis*, serum which also contained PLD-neutralizing antibodies. When Southern blots of *Bam*HI digests of DNA from biovar *ovis* and biovar *equi* isolates of *C. pseudotuberculosis* were probed with pCpO50, bands of 4.8 and 1.9 kilobases, respectively, were seen, suggesting that the genome organization of *pld* is different for isolates from the two biovars.

Corynebacterium pseudotuberculosis is widely distributed among animal populations, causing caseous lymphadenitis in sheep and goats and both ulcerative lymphangitis and pectoral, inguinal, and abdominal abscesses in horses (9, 13, 20, 24, 35). Infections also occur in cattle and humans (17). Isolates of *C. pseudotuberculosis* from domestic animals are phenotypically heterogeneous, but two biovars can be distinguished (4, 29). Most isolates from horses reduce nitrate (94%) and are streptomycin susceptible (biovar *equi*), while those from sheep and goats fail to reduce nitrate (97%) and are streptomycin resistant (biovar *ovis*). Restriction endonuclease analysis of the DNA of these groups has identified differences between isolates from biovars *ovis* and *equi* (12, 29), but the phenotypic and genotypic bases for the differential host preference have not been defined.

The exact mechanism of pathogenesis of *C. pseudotuberculosis* infections is unclear, but the organism produces both a toxic cell wall lipid (7) and an exotoxin (2, 3, 10, 14, 16, 21–23, 25, 29, 30). The exotoxin, which is produced by all known isolates of the organism, is a phospholipase D (PLD); many of its biological properties remain unclear. It is known that PLD hydrolyzes lysophosphatidylcholine and sphingomyelin (2, 29), has a pI of about 9.8, requires calcium and magnesium ions for activity, is toxic for laboratory rodents and domestic animals (14), and lyses sheep erythrocytes in synergy with cholesterol oxidase and phospholipase C (*equi* factors) produced by *Rhodococcus equi* (2, 3, 29). Reported molecular weights of PLD, as determined by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis, vary widely (10, 16, 25). Recent work in our laboratory, in which we have used preparative isoelectric focusing and SDS-polyacrylamide gel electrophoresis with subsequent renaturation of activity, has shown the molecular size to be 31.7 kilodaltons (10; unpublished data). There is no evidence that *C. pseudotuberculosis* produces any hemolytic proteins or other synergistically hemolytic proteins.

Numerous pathogenic bacteria produce phospholipases (6, 8, 15, 18, 33, 34) which may be involved in the pathogenesis of disease (8, 33, 34). Further basic information is needed about the synthesis, regulation, secretion, and mode of action of these enzymes to precisely elucidate their role in disease. Knowledge of the structure and function of the PLD gene (*pld*) will help to determine its role in the pathogenesis of *C. pseudotuberculosis* infections and perhaps the role of other phospholipases in disease. We report here the cloning and partial characterization of *pld* from *C. pseudotuberculosis*.

MATERIALS AND METHODS

Bacteria and culture conditions. *C. pseudotuberculosis* isolate Whetten 1, obtained from a liver abscess in a goat in Arizona, was used as the source of DNA for cloning procedures. This isolate is Pld^+ and is characteristic of *C. pseudotuberculosis* in its fermentation of sugars and other biochemical properties and in the restriction enzyme digest profile of its DNA (29). It was cultivated in brain heart infusion (Difco Laboratories, Detroit, Mich.) with 0.1% Tween 80. Cultures were incubated at 37°C with agitation at 60 rpm for 24 to 36 h. Cells were harvested by centrifugation (5,000 × *g* at 4°C for 20 min) and washed twice with phosphate-buffered saline (0.01 M, pH 7.2). *Escherichia coli* LE392 and cosmid vector pLAFR2 were used in initial cloning experiments. Subcloning was done in pUC19, and recombinants were transformed into *E. coli* DH5α or JM109. *E. coli* was cultivated in Luria-Bertani (LB) broth at 37°C with agitation (200 rpm) for 16 to 24 h or on LB agar plates (1.5% agar) incubated at 37°C (19).

Assays of PLD activity. Recombinants were screened for PLD activity by a synergistic hemolytic assay (10, 29). Sheep erythrocytes (5%) and filtered culture supernatant

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† Arizona Technical Paper no. 4274, a contribution of the Arizona Agricultural Experiment Station, with the approval of the director.

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fluid (10%) from *R. equi* ATCC 33701 (cultivated in brain heart infusion broth) were added to LB agar. Colonies producing PLD after cultivation on this medium were surrounded by a zone of hemolysis after 48 to 72 h of incubation of 37°C.

PLD activity was confirmed by measuring enzymatic release of [^{14}C]choline from labeled sphingomyelin (10, 16, 23). Samples of 10 μl of dilutions of culture supernatant fluid in buffer (25 mM NaCl, 5 mM $MgCl_2$, 100 mM Tris hydrochloride) were incubated at 37°C with 25 μl of [*methyl*-^{14}C]choline sphingomyelin (specific activity of 1,820 cpm/nmol, in the same buffer; Dupont, NEN Research Products, Boston, Mass.). The reaction was stopped with 15 μl of 0.1 N HCl after 30 min of incubation, and choline was separated from sphingomyelin by extraction with chloroform-methanol (1:2) (5). The aqueous phase was analyzed by liquid scintillation counting. Enzyme activity was expressed in units (nanomoles of sphingomyelin hydrolyzed per milliliter per 30 min).

PLD also protects ruminant erythrocytes from lysis by staphylococcal β-hemolysin (2, 22). To assay for this phenomenon, washed sheep erythrocytes (5%) were added to purified agar (BBL Microbiology Systems, Cockeysville, Md.) dissolved in phosphate-buffered saline (0.01 M, pH 6.0, with 300 mosmol of NaCl per liter) and the medium was poured in 10-ml amounts into immunodiffusion plates (45 by 95-mm radius; Miles Laboratories, Inc., Elkhart, Ind.). Wells (5 mm) were cut, and 30-μl samples of culture supernatant fluids from recombinants were added. After 24 h of incubation at 37°C, plates were spread with a thin layer of filtered supernatant fluid from a culture of *Staphylococcus aureus* ATCC 25178. After an additional 4 h of incubation at 37°C, plates were placed at 4°C for 1 to 2 h. Wells containing β-hemolysin-inhibiting activity were surrounded by a zone of intact erythrocytes in a background of lysed erythrocytes.

Preparation of corynebacterial DNA. The cell pellet harvested from a 500-ml culture of isolate Whetten 1 was suspended in 2.5 mM Tris–2.5 mM EDTA–0.5 M sucrose (pH 8.0) with 5 mg of lysozyme (Sigma Chemical Co., St. Louis, Mo.) per ml. After incubation at 37°C for 2 h, the cells 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. The protoplasts were extracted with 10 mM Tris–1 mM EDTA (TE)-saturated phenol, 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, Inc., Gaithersburg, Md.) in TE 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 0.2 M ammonium acetate.

DNA was prepared by the same method from an equine isolate of *S. aureus*, *Arcanobacterium haemolyticum* ATCC 9345, and *C. pseudotuberculosis* isolates 803599 (biovar *equi* caprine isolate from California), BB1, Carne 5, H802268 (biovar *ovis* ovine isolates from Canada, Australia, and Canada, respectively), M3 (biovar *ovis* caprine isolate from Canada), and 22667 and 2808 (biovar *equi* equine isolates from Texas and Arizona, respectively). These preparations were used only in Southern blots, as described below.

Construction of corynebacterial cosmid library. pLAFR2 DNA was prepared by alkaline lysis (19) of *E. coli* LE392(pLAFR2) that was cultivated in LB broth containing 15 μg of tetracycline per ml (Fig. 1). The DNA was purified by centrifugation on a cesium chloride gradient (55,000 rpm, 20°C, 65 VTi rotor; Beckman Instruments, Inc., Fullerton, Calif.) (19). The cosmid DNA was digested with *Bam*HI (Promega Biotec, Madison, Wis.), dephosphorylated with calf intestinal alkaline phosphatase (Promega), phenol-chloroform-isoamyl alcohol extracted, and ethanol precipitated (19). A partial digest of about 500 μg of corynebacterial DNA was prepared with *Sau*3a (Promega) (28). The fragments were sized on a 10 to 40% sucrose gradient by centrifugation for 12 h at 20°C at 39,000 rpm in an SW41 rotor (Beckman Instruments) (19). Fractions (0.5 ml) were collected, and the DNA was precipitated with 95% ethanol and dissolved in TE. Fragments of 17 to 30 kilobases (kb) were retained for construction of a genomic library.

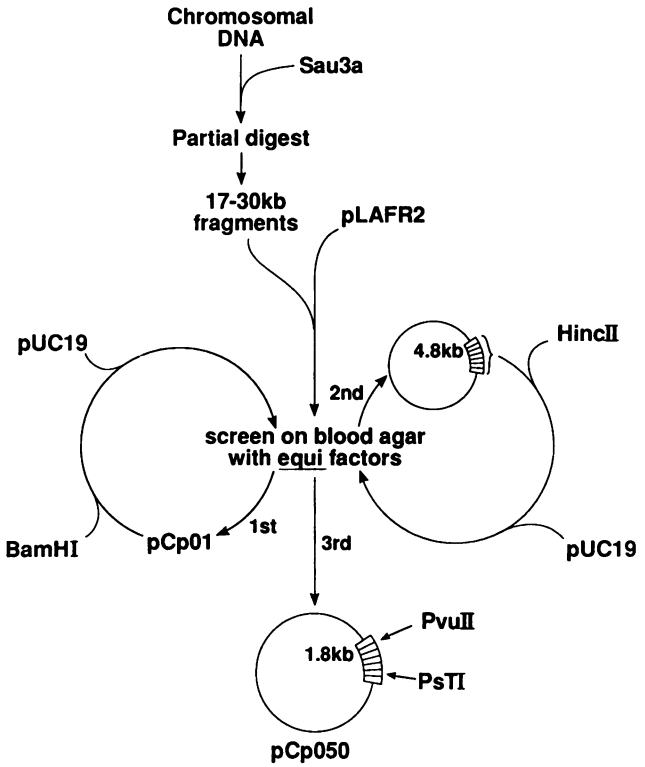

FIG. 1. Schematic diagram depicting the strategy used in cloning of *pld*.

The corynebacterial DNA fragments were mixed with prepared cosmid DNA in a final molar ratio of 3:1 and ligated with T4 DNA ligase (Bethesda Research Laboratories) at 16°C for 12 h. This ligation mixture (10 μl) was packaged with the Packagene system according to the instructions of the manufacturer (Promega).

The cosmid library was screened for PLD production by plating infected cells on LB agar containing 10% *equi* factors, 5% ovine blood, and 15 μg of tetracycline per ml. Plates were incubated in a humidified incubator at 37°C, and hemolytic colonies appeared after 24 to 36 h. Synergistic hemolytic activity of the gene product was confirmed by streaking these recombinants onto blood agar, alone and adjacent to a linear streak of *R. equi* (a modified CAMP test). From the several synergistically hemolytic, putatively Pld$^+$ recombinants, we selected one clone for further work and designated it pCpO1.

The size of the insert in pCpO1 was 24 kb. The insert was isolated by *Bam*HI digestion of pCpO1, followed by agarose gel electrophoresis and electroelution (19). After complete *Bam*HI digestion, fragments of the insert were subcloned

into the *Bam*HI site of pUC19 and transformed into competent *E. coli* JM109. Cells were plated on LB agar with 10% *equi* factors, 5% ovine blood, and 50 μg of ampicillin per ml. Hemolytic colonies appeared after 48 to 72 h of incubation at 37°C. The plasmids in several of these recombinants contained a 4.8-kb insert fragment which was digested with *Hin*cII, ligated into the *Hin*cII site of pUC19, and transformed into *E. coli* DH5α. Transformants were screened for a synergistic hemolytic gene product as described above. Synergistically hemolytic recombinants were shown to contain a 1.8-kb insert in pUC19. This plasmid was designated pCpO50.

Southern blotting. Chromosomal DNA from *C. pseudotuberculosis*, *S. aureus*, and *A. haemolyticum* was digested with *Bam*HI, electrophoresed in 0.7% agarose, and transferred to a nitrocellulose membrane by the method of Southern (32). The probe, pCpO50, was labeled with ^{32}P by the random primer method (11) and hybridized to membranes at 65°C in 6× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.5% SDS–5× Denhardt solution with 100 μg of sonicated calf thymus DNA and 500 μg of dextran sulfate per ml. Filters were washed in 2× SSC–1% SDS at room temperature for 1 h and then in 0.1× SSC–0.1% SDS at 65°C for 1 h. Autoradiography against Kodak X-OMAT AR film was at −70°C.

Electrophoretic examination of native and recombinant proteins. Proteins from cells and culture supernatant fluids of *C. pseudotuberculosis* isolate Whetten 1 (caprine, biovar *ovis*), *E. coli*(pUC19), and *E. coli*(pCpO50) were examined by SDS-polyacrylamide gel electrophoresis, as was native PLD purified by recycling isoelectric focusing. About 50 μg of protein was loaded into each lane, and the gels were run for 4 to 6 h at 175 to 650 V. Gels were fixed overnight in 50% ethanol and 5% glacial acetic acid, followed by color silver staining by a Gelcode (Pierce Chemical Co., Rockford, Ill.) method (27).

Proteins in some unstained gels were electrotransferred to nitrocellulose membranes (Bio-Rad Laboratories, Richmond, Calif.) and probed with sheep serum (see below) diluted 1:25 and a rabbit anti-sheep immunoglobulin G-peroxidase conjugate (Sigma) diluted 1:1,000. Blots were incubated with substrate at room temperature for 15 min (1).

Anti-PLD serum. Serum containing PLD-neutralizing antibodies was obtained from a sheep with chronic *C. pseudotuberculosis* infection. Four weeks before the serum was collected from this sheep, 5 ml of formalinized supernatant fluids from a culture of isolate Whetten 1 was administered intravenously to boost the anti-PLD antibody titer. Serum was harvested from coagulated samples of peripheral blood and stored at −20°C. This serum contained antibodies which neutralized the activity of native and recombinant PLDs, as measured by the radiometric assay and by inhibition of synergistic hemolysis.

RESULTS AND DISCUSSION

Cloning of *pld*. We constructed a genomic library in the cosmid vector pLAFR2 and screened this library for PLD production in *E. coli* (Fig. 1). DNA isolated from clones expressing synergistic hemolytic activity contained 24-kb inserts of corynebacterial DNA. To locate *pld*, we subcloned this fragment into pUC19 and employed the same screen on blood agar plates with *equi* factors. Plasmids from hemolytic colonies contained a 1.8-kb insert, and based on estimates of the molecular weight of PLD, this is more than sufficient to span the structural gene and accessory sequences. One of these clones, designated pCpO50, was further characterized.

TABLE 1. Sphingomyelinase and other activities in cells and supernatant fluids from cultures of *C. pseudotuberculosis*, *E. coli*(pCpO50), and *E. coli*(pUC19)

Culture	PLD activity[a]		SH activity[d]	βHI activity[e]
	CSF[b]	Cell extract[c]		
E. coli(pUC19)	0	0	0	0
E. coli(pCpO50)	186	445	10	10
C. pseudotuberculosis	158	25	1	10

[a] Measured by radiometric assay as nanomoles of sphingomyelin hydrolyzed per 30 min per milliliter.
[b] Culture supernatant fluid. Prepared as described in text.
[c] Prepared as described in text.
[d] Synergistic hemolytic activity in culture supernatant fluid, expressed as zone diameter in millimeters. Measured as described in text.
[e] Staphylococcal β-hemolysin-inhibiting activity in culture supernatant fluid, expressed as zone diameter in millimeters. Measured as described in text.

Function of cloned PLD. *E. coli* DH5α(pCpO50), *E. coli* DH5α(pUC19), and *C. pseudotuberculosis* isolate Whetten 1 were cultivated as described above. Cells were harvested by centrifugation, and supernatant fluids were filtered (220-nm pore diameter). Supernatant fluids were examined for β-hemolysin-inhibiting, synergistic hemolytic, and sphingomyelinase activities as described above. Cell pellets were suspended in 5 ml of TE (pH 8.0) and extracted with 5 ml of chloroform. After centrifugation (5,000 × *g*, 5 min, 4°C), the aqueous fraction was collected and both this and the culture supernatant fluid were examined for sphingomyelinase activity by the radiometric assay. Results (Table 1) demonstrated that no PLD activity was found in *E. coli*(pUC19) but that supernatant fluids and cell extracts from *E. coli* (pCpO50) and *C. pseudotuberculosis* contained enzyme activity. In *C. pseudotuberculosis*, enzyme activity was found primarily in culture supernatant fluids, while in *E. coli*(pCpO50), activity in extracts was nearly 2.5 times greater than that in culture supernatant fluids. This suggested that although native PLD was secreted by *C. pseudotuberculosis*, recombinant PLD was found primarily in the periplasm and cytoplasm of *E. coli*(pCpO50). Supernatant fluids from cultures of *E. coli*(pCpO50), *E. coli*(pCpO1), and Whetten 1 but not from *E. coli*(pUC19) exhibited both synergistic hemolytic and β-hemolysin-inhibiting activities (Table 1), both of which are characteristic of PLD (2, 10, 14). Enzyme activity in supernatants of cultures of both *C. pseudotuberculosis* and *E. coli*(pCpO50) were neutralized by the sheep antiserum.

Cell extracts and culture supernatant fluids produced as described above were combined and examined by SDS-polyacrylamide gel electrophoresis and Western blotting (immunoblotting). A major product of *E. coli*(pCpO50) labeled by sheep anti-PLD antibodies had a molecular size of about 31 kilodaltons (Fig. 2). Many proteins in supernatant fluids from a culture of *C. pseudotuberculosis* isolate Whetten 1 were labeled by sheep antibodies, including one of about 31,000 M_r (Fig. 2). Purified native PLD also contained only a single antibody-reactive band, a protein of about 31,000 M_r. The putative recombinant PLD apparently has a slightly higher molecular weight than native PLD, possibly owing to the presence of an uncleaved signal sequence. No proteins from *E. coli*(pUC19) were recognized by these antibodies.

The bands of approximately 31 kilodaltons in culture supernatant fluids from the native organism and from *E. coli*(pCpO50) compare closely with the reported molecular

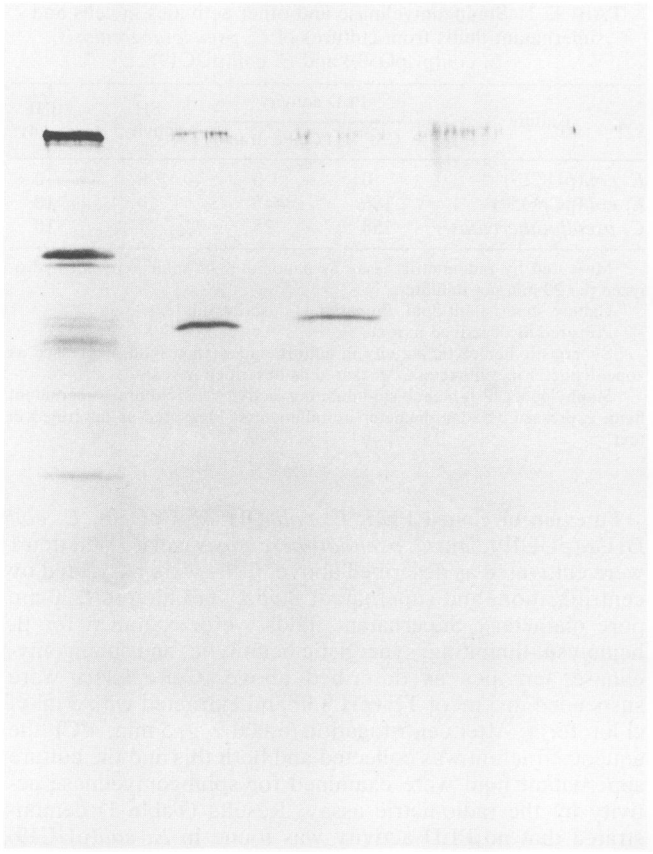

FIG. 2. Western blot of native and recombinant proteins probed with sheep antiserum. Left to right: Culture supernatant fluid from *C. pseudotuberculosis*; PLD partially purified by recycling isoelectric focusing; culture supernatant fluid from *E. coli*(pCpO50); culture supernatant fluid from *E. coli*(pUC19); and loading buffer.

weight of PLD (10, 14, 16; unpublished data). Based on (i) production of PLD (as measured by three different assays) by *E. coli*(pCpO50), (ii) neutralization of native and recombinant PLD by the sheep antiserum, (iii) labeling of native and recombinant proteins of nearly identical molecular weight by the antiserum, and (iv) lack of evidence for other hemolytic, synergistically hemolytic, or sphingomyelin-hydrolyzing proteins in *C. pseudotuberculosis*, the 31-kilodalton protein produced by *E. coli*(pCpO50) must be PLD.

Some workers have speculated on the relationship between the enzymatic and staphylococcal β-hemolysin-inhibiting activities of PLD, and one group suggested that the two activities are properties of separate molecules (22); alternatively, it may be that the PLD radioassay detects only enzymatically active exotoxin, whereas the β-hemolysin inhibition test detects both active and inactive exotoxin (23). The recombinant protein produced by *E. coli*(pCpO50) has both enzyme and β-hemolysin-inhibiting activities, suggesting that both properties are expressed by a single molecule. Although it is still possible that two proteins are produced from the cloned corynebacterial DNA fragment, it would require (i) that antibodies capable of neutralizing β-hemolysin-inhibiting activity are incapable of labeling the β-hemolysin-inhibiting molecule in a Western blot and (ii) the presence of two open reading frames in the cloned DNA. Sequence analysis of the cloned DNA will answer with finality the question of the separability or inseparability of enzyme and β-hemolysin-inhibiting activities.

Genetic organization of *pld*. The restriction map of the 1.8-kb insert in pCpO50 (Fig. 3) contains restriction sites for *Pvu*II and *Pst*I. There were no restriction sites for *Eco*RI, *Hin*dIII, *Cla*I, *Bgl*I, *Bgl*II, *Sal*I, *Bam*HI, *Eco*RV, *Kpn*I, *Hin*cII, *Xho*I, *Xba*I, *Sma*I, *Sph*I, and *Bcl*I. The location of *pld* on this fragment has not been determined. However, attempts to subclone *Pst*I digests of cosmid clones did not yield synergistically hemolytic recombinants, suggesting that the *Pst*I site in the insert is in the structural gene or in an important regulatory sequence. No attempts were made to subclone *Pvu*II digests.

As noted, the two biovars of *C. pseudotuberculosis* can be distinguished phenotypically by their ability to reduce nitrate or response to streptomycin and genotypically by restriction fragment length polymorphisms (29). To examine the organization of *pld* in isolates from biovars *ovis* and *equi*, we probed Southern blots of *Bam*HI-digested chromosomal DNA from clinical isolates of *C. pseudotuberculosis* with labeled pCpO50. In isolates belonging to biovar *ovis*, a single band of about 4.8 kb was observed (Fig. 4), a result consistent with the subcloning of *pld* on a 4.8-kb *Bam*HI fragment from pCpO1. However, in DNA from biovar *equi* isolates, the probe identified a 1.9-kb band (Fig. 3). No homology with chromosomal DNA from *S. aureus* or *A. haemolyticum* was demonstrated.

These results suggest that the PLD gene is present in single copy, although sequence data on the cloned fragment and reprobing of chromosomal DNA with an internal fragment of *pld* will be necessary to demonstrate this conclusively. The lack of homology between *pld* and chromosomal DNA from *S. aureus* and *A. haemolyticum* was not surprising. *S. aureus* produces a sphingomyelinase C (β-hemolysin) (8) and *A. haemolyticum* produces a PLD (26), but there is no evidence of antigenic similarity between either of these enzymes and PLD from *C. pseudotuberculosis* (unpublished data). Further, when restricted DNA from *C. pseudotuberculosis* was probed with the cloned *S. aureus* β-hemolysin gene, no corynebacterial DNA sequences were labeled (unpublished data).

An unexpected finding was the different hybridization pattern of pCpO50 with chromosomal DNA from isolates in biovars *ovis* and *equi*. There is no evidence that PLD produced by isolates from biovar *equi* is different from that produced by isolates from biovar *ovis* (10), and we have no evidence for differences in the PLD gene in isolates of the two biovars. However, there is apparently at least one

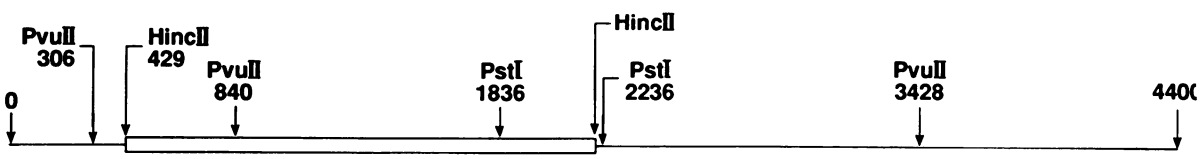

FIG. 3. Map showing restriction sites in the corynebacterial DNA fragment in pCpO50.

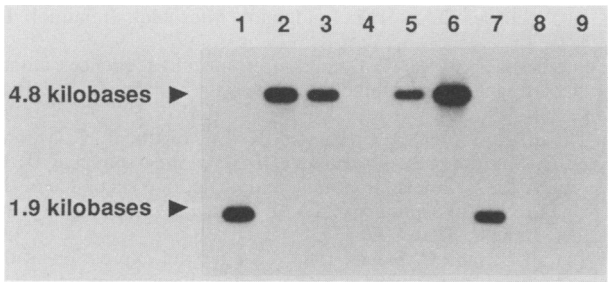

FIG. 4. Southern blot of bacterial chromosomal DNA probed with ^{32}P-labeled pCpO50. Lanes: 1, isolate 803599 (caprine, biovar *equi*); 2, isolate BB1 (ovine, biovar *ovis*); 3, isolate Carne 5 (ovine, biovar *ovis*); 4, *S. aureus*; 5, isolate M3 (caprine, biovar *ovis*); 6, isolate H802268 (ovine, biovar *ovis*); 7, isolate 22667 (equine, biovar *equi*); 8, isolate 2808 (equine, biovar *equi*); 9, *A. haemolyticum* ATCC 9345.

additional *Bam*HI restriction site in DNA flanking *pld* in biovar *equi* isolates.

In the absence of a defined phenotypic or genotypic basis for the host preference of organisms in the two biovars of *C. pseudotuberculosis*, it has been tempting to speculate about the possible role of nitrate reductase as a marker of such virulence attributes. The demonstrated difference in DNA sequences flanking *pld* provides an additional temptation. While the differences may be inconsequential, they could contain *pld*-linked genes responsible for host specificity. Comparison of *pld* and its gene product from biovar *equi* isolates with *pld* from biovar *ovis* isolate Whetten 1 and the recombinant gene product should provide answers to these questions.

Cloning of *pld* will allow us to study the PLD molecule in greater detail and to examine the role of PLD in the pathogenesis of *C. pseudotuberculosis* infections. Comparison of *pld* with genes for phospholipases from other bacteria may contribute to understanding of structure-function relationships in this group of enzymes.

ACKNOWLEDGMENTS

We thank Neal Groman, Steve Gill, and Ken Bayles for helpful discussions.

This work was supported by funds from the U.S. Department of Agriculture (Project numbers 87-CRSR-2-3118, 88-CRSR-34116-3750, and ARZT-174861-H-02-06), by BRSG S07 RR07002 awarded by the Biomedical Research Support Grant Program, Division of Research Resources, National Institutes of Health, and by Public Health Service grant R37-AI17474 from the National Institute of Allergy and Infectious Diseases.

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