INFECTION AND IMMUNITY, Oct. 1987, p. 2348–2354
0019-9567/87/102348-07$02.00/0

Vol. 55, No. 10

Identification and Characterization of the *Pasteurella haemolytica* Leukotoxin

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Received 6 April 1987/Accepted 26 June 1987

The identification and chromatographic characterization of the leukotoxin of *Pasteurella haemolytica* is described. The toxin, which has an apparent native molecular weight of greater than 400,000 as judged by gel exclusion chromatography, has a 105-kilodalton (105K) polypeptide as its major protein component. The proteolytic degradation of the 105K polypeptide could be correlated with the loss of toxin activity in aging cultures of *P. haemolytica*. Antisera raised against purified 105K polypeptide neutralized toxin activity. A 3.9-kilobase-pair fragment of the *P. haemolytica* genome cloned into a plasmid vector resulted in the production of intracellular toxin in *Escherichia coli* host cells. The restriction map of this clone shows significant overlap with the map of a previously reported leukotoxin clone (R. Y. C. Lo, P. E. Shewen, C. A. Strathdee, and C. N. Greer, Infect. Immun. 50:667–671, 1985). Finally, antisera raised against the 105K species labeled the *P. haemolytica* cell surface in a nonuniform, punctate manner.

Pasteurella haemolytica is the pathogenic agent in pneumonic pasteurellosis, which is a principal factor in the morbidity and mortality associated with the complex syndrome called shipping fever. This organism secretes a toxin which kills the leukocytes of cattle, goats, and sheep but not those of nonruminants (5, 12). Since phagocytic cells are considered to be a major mechanism of host defense against respiratory infections, the *P. haemolytica* leukotoxin is likely to be a major virulence factor.

There have been several reports of the identification of the *P. haemolytica* toxin from culture supernatants. Himmel et al. (10) purified a protein with a native molecular weight of 150,000 as judged by gel filtration and described its dissociation into 20,000- and 50,000-molecular-weight components in the presence of sodium dodecyl sulfate (SDS). However, their most purified preparations killed less than 1% of bovine alveolar macrophage cells after a 6-h exposure by using trypan blue exclusion. Baluyut et al. (2) claimed, on the basis of ultrafiltration, that the leukotoxin has a native molecular weight greater than 300,000 but did not characterize the material at the molecular level. Lo et al. (13) reported the cloning of a *P. haemolytica* DNA fragment, the expression of which yielded leukotoxin activity in *Escherichia coli* cell lysates. Mosier et al. (17) reported that the native cytotoxin has a molecular weight of 160,000 as judged by gel filtration chromatography. This material yielded a number of distinct polypeptide species when subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE). In this report, we demonstrate that the toxin has a single polypeptide component, with an apparent molecular weight of 105,000, as judged by SDS-PAGE, which has a primary proteolytic breakdown product with a molecular weight of 95,000 both in *P. haemolytica* culture supernatants and within *E. coli* cells carrying the cloned toxin gene.

MATERIALS AND METHODS

Bacterial strains, media, and culture conditions. Representative *P. haemolytica* strains from each of the 15 serotypes (7) were obtained courtesy of E. L. Biberstein, University of California, Davis. Biotype A, serotype 1 strains were obtained from the Texas Veterinary Diagnostic and Laboratory System in College Station and Amarillo, Tex. *P. haemolytica* cultures were grown in brain heart infusion (BHI; Difco Laboratories), Dulbecco minimal essential medium (DMEM) supplemented with 10% fetal calf serum (FCS), or a dialysate (DBHI) obtained by dialyzing 10-fold-concentrated BHI against 9 volumes of distilled water. LB-Amp was Luria broth (16) supplemented with ampicillin (50 μg/ml). Minimal glycerol medium was M9 minimal medium (16) with 0.2% glycerol as a carbon source.

SDS-PAGE and Western blotting. SDS-PAGE was performed as previously described (1). Western blotting (immunoblotting) was performed exactly as described by Towbin et al. (22). Immunoreactive bands were visualized with a second antibody conjugated to horseradish peroxidase (9).

Leukotoxin assay. The continuous bovine lymphoma cell line BL-3 obtained from G. Theilen, University of California, Davis, was used to estimate leukotoxin activity. BL-3 cells undergo characteristic morphological changes upon exposure to active leukotoxin (Fig. 1). In a standard assay, 10^6 cells were incubated with toxin samples (less than 50 μl) for 1 hour at 37°C in 1 ml of growth medium, which was Leibovitz L-15 Medium with L-glutamine (Gibco Laboratories) supplemented with 10% FCS. It was observed that certain lots of FCS were inhibitory to toxin activity. Some assays were performed in phosphate-buffered saline (8 g of NaCl, 0.2 g of KCl, 1.15 g of Na_2HPO_4, 0.2 g of KH_2PO_4, and 1 g of glucose per liter, supplemented with 0.5% bovine serum albumin and 1 mM $CaCl_2$). The calcium was found to be required for leukotoxin activity at low concentrations of toxin and could not be replaced by magnesium, manganese, or zinc. One unit of toxin activity is defined as the minimal amount of toxin, as determined by serial dilution, required to produce the morphological change in >95% of the input BL-3 cells. This simple visual assay correlates well with more tedious assays based on the killing of freshly isolated bovine macrophages, leukocytes, and neutrophils (2, 4, 18), as well as with a similar assay based on neutral red uptake of BL-3 cells described by Greer and Shewen (8).

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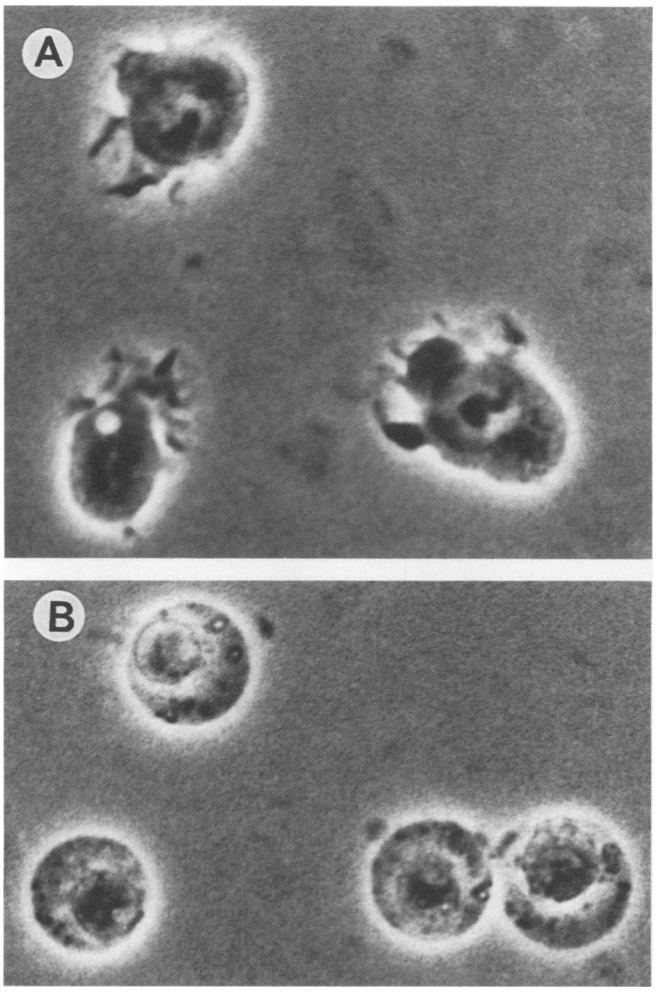

FIG. 1. Visual assay of leukotoxin activity by using bovine lymphoma (BL-3) cells. Cells were incubated in the absence (A) or presence (B) of leukotoxin as described in Materials and Methods. Phase-contrast micrograph magnification, ×250.

Source of neutralizing antibodies. Antibodies from cattle experimentally infected with live *P. haemolytica* were obtained from R. Loan, Department of Veterinary Microbiology and Parasitology, College of Veterinary Medicine, Texas A&M University. Antibodies were also raised in New Zealand White male rabbits immunized with Formalin-killed *P. haemolytica*. In addition, antibodies specific for the 105-kilodalton (105K) polypeptide species secreted by *P. haemolytica* were raised in New Zealand White male rabbits by immunization with material purified by preparative SDS-PAGE. Gel slices containing the 105-kilodalton band were directly emulsified with Freund adjuvant and injected subcutaneously into rabbits.

Immunological techniques. Immunoprecipitation of culture supernatants and French pressates and analysis of SDS-polyacrylamide gel were done as previously described (15). Immunoprecipitation of whole-cell extracts were done essentially as described by Stader et al. (20). Briefly, 1 ml of a saturated culture of cells was centrifuged for 2 min in an Eppendorf Microfuge (Beckman Instruments, Inc.), and the pellet was suspended in 50 μl of 1% SDS–1 mM EDTA (ethylenediaminetetraacetic acid), pH 7.8. This suspension was boiled for 1 min, supplemented with 0.65 ml of TSET buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 1 mM EDTA, and 2% Triton X-100), and centrifuged again for 10 min at room temperature in the microfuge. The insoluble pellet was removed with a toothpick, and the supernatant was immunoprecipitated as described above. Indirect immunofluorescent staining of *P. haemolytica* was performed by using fresh, logarithmic-phase cells grown in BHI. The cells from 50 μl of culture were washed three times in sterile 0.15% NaCl. After the last wash, the cells were resuspended in 50 μl of undiluted antiserum and incubated at 37°C for 30 min. After incubation, the cells were washed three times with PBS. For staining, the cells were resuspended in buffer containing fluorescein-conjugated goat anti-rabbit immunoglobulin G (heavy and light chain specific; Cappel Laboratories) and incubated at 37°C for 0.5 h. After the incubation, the cells were washed with phosphate-buffered saline three times and were immobilized on an agar-coated microscope slide. Microscopy was performed with a Leitz Dialux 20 microscope equipped with phase-contrast optics and epi-illumination for fluorescence.

Preparation of crude leukotoxin. *P. haemolytica* biotype A, serotype 1 was grown in DBHI to an A_{550} of 4, and the culture supernatant was collected by low-speed centrifugation. The supernatant was concentrated 10-fold by ultrafiltration by using an Amicon DC-2 hollow-fiber apparatus with a molecular weight cutoff of 30,000. Tris base (20 mM, 2 liters) adjusted to pH 8.0 with HCl was added, and the mixture was concentrated 20-fold by ultrafiltration. This step was necessary to achieve the salt and pH requirements of DEAE ion-exchange chromatography, since dialysis at this stage resulted in the loss of toxin activity. The concentrated supernatant was subjected to ultracentrifugation at 117,000 × *g* for 1 h at 4°C to remove insoluble material. This preparation contained typically 5,000 U/ml and was used as the crude toxin preparation in all chromatography experiments.

Cloning of toxin gene. *P. haemolytica* chromosomal DNA was prepared by the method of Silhavy et al. (19) and partially digested with *Sau*3A. The digest was fractionated by sedimentation in neutral sucrose (14), and fractions containing 3- to 10-kilobase-pair fragments, as judged by agarose gel electrophoresis, were pooled and concentrated by alcohol precipitation to a final level of 100 μg/ml. The omega-complementing plasmid pHG165 was chosen as the library vector. Plasmid vector pHG165 (10 μg) (21) was cleaved with *Bam*HI and treated with alkaline phosphatase to remove terminal phosphates. After phenol extraction and concentration by ethanol precipitation, the vector DNA was mixed with the 3- to 10-kilobase-pair *P. haemolytica* DNA at

TABLE 1. Neutralization of leukotoxin activity[a]

Antiserum source	Neutralization at antibody dilution:						
	1	1:2	1:4	1:8	1:16	1:32	1:64
Cow	−	−	−	−	−	±	+
Rabbit	−	−	−	−	±	+	+
Rabbit (preimmune)	+	ND	ND	ND	ND	ND	ND

[a] A 10-μl sample of partially purified toxin (100 U) was mixed with 10-μl of serially diluted antiserum obtained from experimentally infected cattle or from a rabbit immunized with the 105K polypeptide which had been purified by preparative SDS-PAGE. After a 1-h incubation at 37°C, 10^6 BL-3 cells in 1 ml of L-15 growth medium was added to each mixture and toxin activity was assessed as described in Materials and Methods. +, >95% of the BL-3 cells showed morphological changes induced by leukotoxin; −, complete neutralization of leukotoxin; ND, not done.

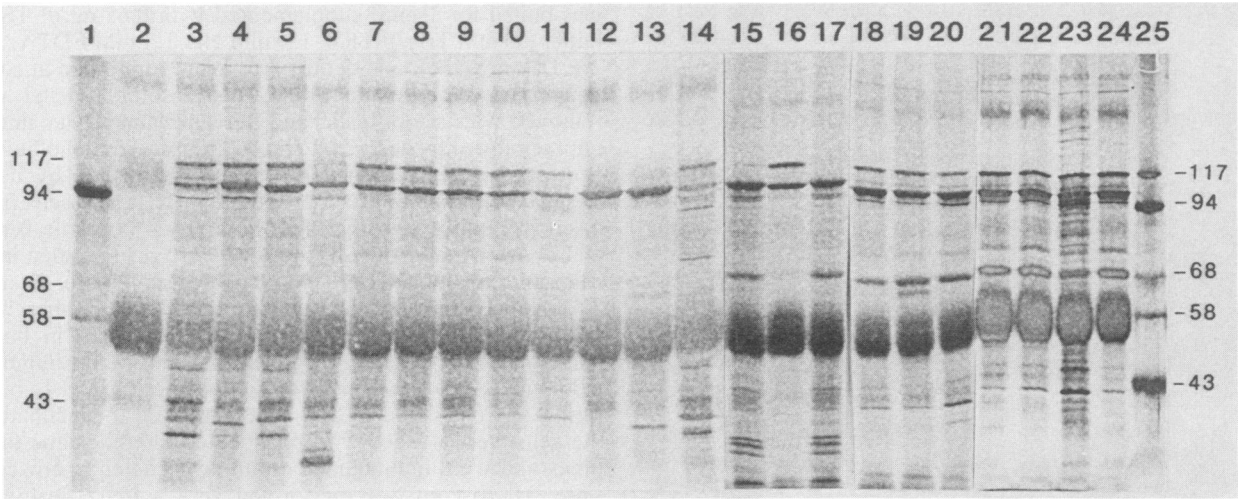

FIG. 2. Immunoprecipitation of culture supernatants from different *P. haemolytica* serotypes. *P. haemolytica* cultures were grown in BHI medium supplemented with 10% FCS. Late logarithmic-phase supernatant (300 μl) was immunoprecipitated with 10 μl of bovine antiserum and analyzed on a polyacrylamide gel with silver staining as described in Materials and Methods. Molecular size is given in kilodaltons. Lanes: 1 and 25, molecular weight standards; 2, control immunoprecipitation by using 300 μl of growth medium; 3 to 17, *P. haemolytica* serotypes 1 to 15; 18 to 24, different clinical field isolates of *P. haemolytica* biotype A, serotype 1. The major diffuse band at approximately 50 kilodaltons is the immunoglobulin G heavy chain added in the immunoprecipitation.

a molar ratio of 1:5 and treated with T4 DNA ligase for 16 h at 18°C. The ligation mixture was transformed into alpha-complementing *E. coli* TB1 *hsdR* (11), and transformants were selected on LB-Amp with 100 μg of X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) per ml. Transformants were obtained at approximately 1,000 colonies per μg of input DNA, approximately 80% of which were colorless and thus contained insert DNA. Immunodetection of the production of the *P. haemolytica* leukotoxin was done by the method of Hawkes et al. (9) with antisera from rabbits immunized with gel-purified 105K antigen (see below) and from cattle experimentally infected with *P. haemolytica*. Positive clones were cultured in LB-Amp and checked for the production of the 105K antigen by Western blotting and immunoprecipitation. To test for toxin activity in the clones, 25-ml cultures grown in LB-Amp were concentrated fivefold in growth medium by centrifugation and lysed by a single passage through a French press at 16,000 lb/in^2. The lysate was cleared of unlysed cells by centrifugation at 3,000 × *g* for 5 min and used in serial dilutions in the BL-3 assay (see above).

RESULTS

105K polypeptide toxin component. The supernatant fluids of *P. haemolytica* biotype A, serotype 1 cultures grown in either of two commonly used growth media, BHI or DMEM-FCS, had comparable levels of leukotoxin activity (200 U/ml for DMEM and for 400 U/ml for BHI) as judged by the BL-3 cytotoxicity assay. This toxin activity was inactivated by antiserum obtained from cattle infected with live *P. haemolytica* cells (Table 1). Immunoprecipitation with this antiserum of the culture supernatants from a number of clinical isolates of *P. haemolytica* biotype A, serotype 1, which is most commonly associated with pneumonic pasteurellosis, and from other serotypes revealed a number of major bands (Fig. 2). The major common feature was the 105K species, which we provisionally identified as a component of the toxin.

On the basis of the band intensity in analyses such as the one shown in Fig. 2, we estimate that approximately 0.5 μg of 105K protein per ml was present in supernatants from cultures in late logarithmic phase. Initial attempts to purify the toxin from BHI and DMEM-FCS failed, because these media contain gram quantities of nondialyzable, ammonium-sulfate-precipitable material. Instead, it was found that DBHI can serve as an acceptable growth medium, typically yielding 1,000 U of toxin activity per ml in the late log phase (Fig. 3A). Overnight aeration of the saturated culture resulted in the loss of the toxin activity, as reported previously (2). Immunoprecipitation with the bovine antibody shows that the kinetics of the accumulation of the 105K species paralleled the time course of toxin production (Fig. 3B). Accompanying the loss of toxin activity during the overnight aeration was the degradation of the 105K species into lower-molecular-weight immunoreactive forms (Fig. 3B, lane 10). Proteolytic degradation of the 105K species was also seen in BHI and DMEM-FCS, with the principal degraded form a 95K species (data not shown).

Toxin neutralization by anti-105K antibodies. Antibodies were raised in rabbits against the 105K polypeptide purified by preparative SDS-PAGE. These antibodies neutralized toxin activity (Table 1) and showed a single precipitin line in Ouchterlony immunodiffusion gels (data not shown). Western blots using this antiserum demonstrated not only that a single supernatant band was labeled, but also that the cell pellet contained another immunoreactive species at 95 kilodaltons (Fig. 4).

Chromatographic behavior of toxin. Supernatant from a late log-phase culture was subjected to several kinds of chromatographic analysis. Toxin activity eluted from DEAE-cellulose at 0.3 to 0.4 M NaCl (Fig. 5A), and SDS-PAGE analysis of the column fractions revealed that the 105K species was also eluted in the same fractions (Fig. 5B).

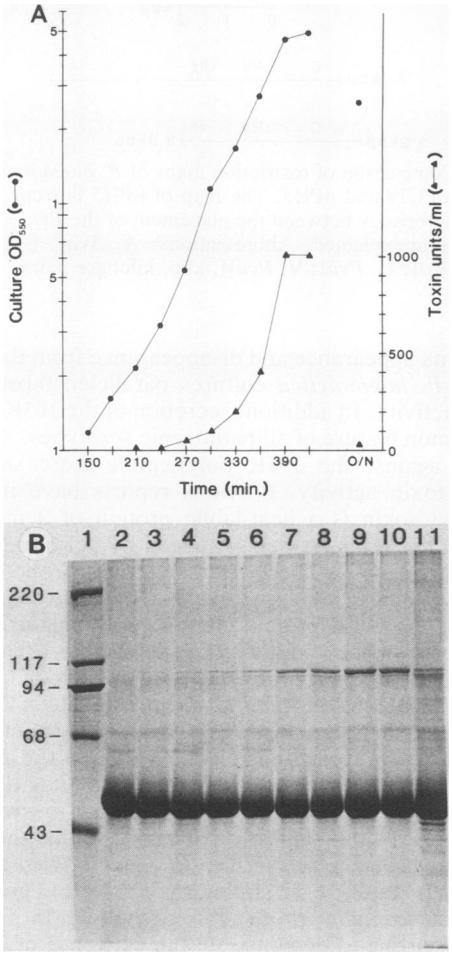

FIG. 3. Kinetics of leukotoxin production and the appearance of the 105K species during culture growth. *P. haemolytica* cells were grown in DBHI, and samples were removed at various times. The samples were assayed for leukotoxin activity and for the presence of the 105K polypeptide as described in Materials and Methods. (A) Kinetics of culture growth and leukotoxin production. (B) Kinetics of 105K polypeptide synthesis. Lanes: 1, molecular weight standards; 2 to 11, immunoprecipitation by using 10 μl of the bovine antiserum and 300 μl of DBHI (lane 2) or 300 μl of culture supernatant from samples taken from a culture at 180 min (lane 3), 210 min (lane 4), 240 min (lane 5), 270 min (lane 6), 300 min (lane 8), 330 min (lane 9), 360 min (lane 10), and 24 h (lane 11).

Neither toxin activity nor the 105K species was bound to carboxymethyl-cellulose at pH 6.5 or to octyl-Sepharose at pH 8.0 in 1 M $(NH_4)_2SO_4$ (data not shown). In addition, the 105K polypeptide and toxin activity showed apparent molecular weights greater than 400,000 based on gel filtration chromatography (Fig. 6).

Identification of cloned toxin gene product. Plasmid libraries of *P. haemolytica* genomic DNA were screened with the bovine and rabbit antisera, and one clone, containing the plasmid pYFC19 with a 3.9-kilobase-pair insert, was shown to react with both antisera and to produce intracellular toxin activity (approximately 50 U/ml of overnight culture [data not shown]). A 105K polypeptide could be immunoprecipitated from lysates of the clone. In addition, lower-molecular-weight species, especially a 95K polypeptide, were immunoprecipitated at various proportions of the 105K polypeptide, depending on the manner of cell disruption

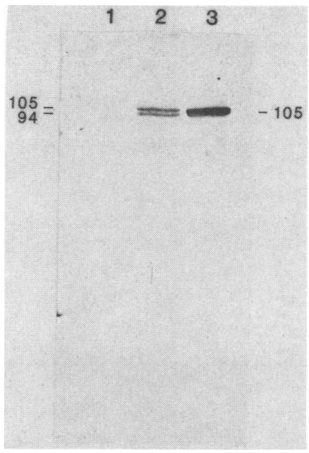

FIG. 4. Western blot analysis by using the anti-105K antibody. The rabbit antiserum prepared against the gel-purified 105K species was used at a 1:200 dilution. Lanes: 1, DBHI control; 2, cellular proteins; 3, culture supernatant.

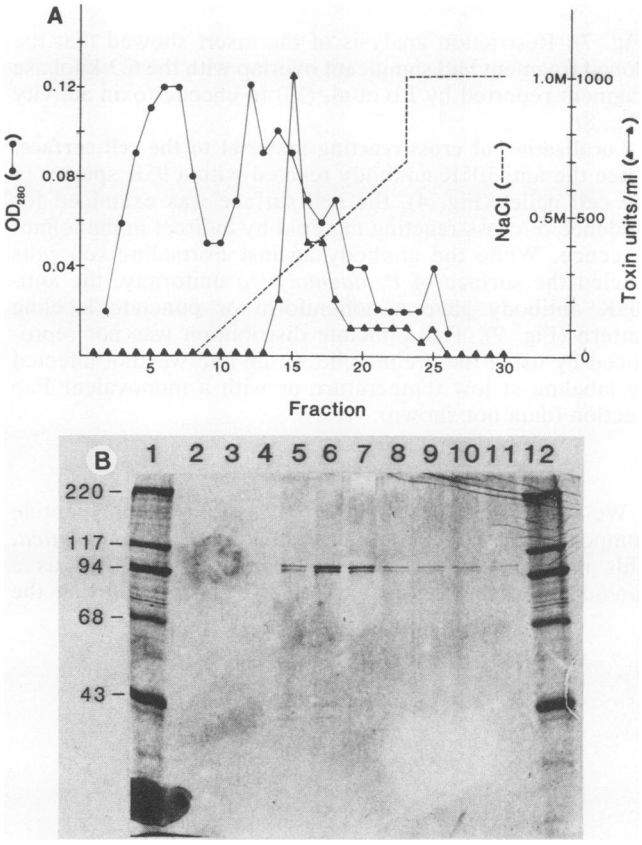

FIG. 5. Elution of leukotoxin activity and the 105K species from DEAE-cellulose. Crude leukotoxin prepared as described in Materials and Methods was applied to a DEAE-cellulose column (1 by 8 cm) in 20 mM Tris, pH 8.0. Elution was accomplished with a linear salt gradient (0 to 0.7 M NaCl). (A) Each fraction (6 ml) was assayed for toxin activity by serial dilution. Approximately 70% of the applied toxin activity was recovered. (B) Samples from selected fractions were analyzed by SDS-PAGE. Lanes 1 and 12 contain molecular weight standards. Lanes 2 to 11 contain fractions 11 to 20, respectively.

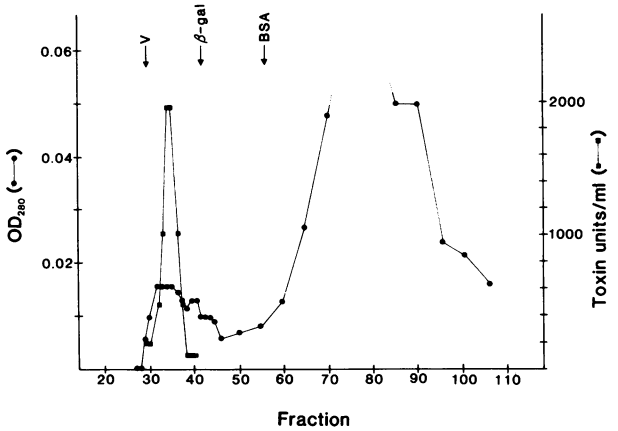

FIG. 6. Gel permeation chromatography of the leukotoxin. Crude leukotoxin prepared as described in Materials and Methods was applied to a Sephacryl S-300 column (2.5 by 45 cm) and eluted with 20 mM Tris, pH 8.0. Each fraction (2.5 ml) was assayed for leukotoxin activity by serial dilution. Approximately 60% of the applied leukotoxin activity was recovered. Fractions 41 to 110 contained less than 50 U of leukotoxin activity per ml. OD_{280}, Optical density at 280 nm; V, void volume; β-gal, β-galactosidase; BSA, bovine serum albumin.

(Fig. 7). Restriction analysis of the insert showed that the cloned fragment had significant overlap with the 6.3-kilobase fragment reported by Lo et al. (13) to encode toxin activity (Fig. 8).

Localization of cross-reacting material to the cell surface. Since the anti-105K antibody reacted with a 95K species in the cell pellet (Fig. 4), the cell surface was examined for evidence of cross-reacting material by indirect immunofluorescence. While the antibody against Formalin-fixed cells labeled the surface of *P. haemolytica* uniformly, the anti-105K antibody gave a nonuniform or punctate labeling pattern (Fig. 9). This punctate distribution was not reproduced by using the preimmune serum and was not affected by labeling at low temperature or with a monovalent Fab fraction (data not shown).

DISCUSSION

We identified a 105K polypeptide as the sole polypeptide component of the leukotoxin produced by *P. haemolytica*. This molecular species was preliminarily identified as a candidate for the toxin based on the observation that the kinetics of its appearance and disappearance from the supernatants of *P. haemolytica* cultures paralleled those of the cytotoxin activity. In addition, secretion of the 105K species was a common feature of all pathogenic serotypes. Antibodies raised against the 105K polypeptide were shown to neutralize toxin activity. Previous reports have indicated that the cytotoxin is a heat-labile protein of a molecular weight of 150,000 or greater as judged by gel filtration (10) and ultrafiltration (2). These results can be reconciled with our present findings by assuming that the active toxin is a multimer of the 105K protein identified on denaturing gels. In fact, our preliminary attempts to purify the toxin by gel filtration suggest that its molecular weight is much greater than 400,000 (Fig. 6). More difficult to reconcile is the claim that the cytotoxin is composed of 20K and 50K subunits (10). This apparent contradiction might be due to the fact that cytotoxin activity is unstable (Fig. 1) and that the 105K protein we identified as the toxin is subject to proteolytic degradation. Previous reports have indicated that there is an extracellular protease released by *P. haemolytica* and also that the cells autolyse in stationary phase (6), potentially releasing intracellular proteolytic activities. In fact, the studies purporting to demonstrate the existence of 20K and 50K subunits were performed by using preparations derived from culture supernatants having variable amounts of low-molecular-weight material which was antigenically similar to the high-molecular-weight (150K) form of the toxin (10). In our hands, the primary breakdown product in culture supernatants was a 95K polypeptide (Fig. 3B), although subsequent degradation to lower-molecular-weight immunoreactive forms has also been seen (data not shown).

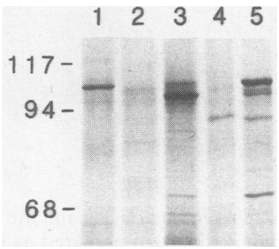

FIG. 7. Immunoprecipitation of cloned leukotoxin produced in *E. coli*. Immunoprecipitations were done with 10 μl of the bovine antiserum and 300 μl of *P. haemolytica* culture supernatant (lane 1), 20 μl of French-pressed cell lysates from cultures grown in LB-Amp (lanes 2 and 3), or whole-cell extracts from cultures grown in minimal glycerol medium (lanes 4 and 5) prepared as described in Materials and Methods. Lanes: 2 and 4, *E. coli* TB1pHG165; 3 and 5, *E. coli* TB1pYFC19. Molecular sizes are shown in kilodaltons.

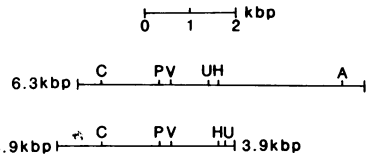

FIG. 8. Comparison of restriction maps of *P. haemolytica* DNA inserts in pYFC19 and pPH5. The map of pPH5 is from Lo et al. (13). The discrepancy between the placement of the *Pvu*I site in the two maps is unexplained. Abbreviations: A, *Ava*I; C, *Cla*I; H, *Hin*dIII; P, *Pst*I; U, *Pvu*I; V, *Pvu*II; kbp, kilobase pairs.

Our data support the idea that the 105K species is the only protein component of the active toxin. The size of the cloned *P. haemolytica* DNA fragment which encodes the toxin protein is only about 0.9 kilobase pairs larger than the minimum required for a polypeptide of this size. Nevertheless, the toxin synthesized in *E. coli* was apparently full length and fully active, indicating that no modification specific to *P. haemolytica* was required. In addition, the most highly purified fractions from ion-exchange chromatography show only the 105K species and the 95K breakdown product (Fig. 5B).

When whole-cell extracts of *P. haemolytica* were subjected to Western blot analysis by using the 105K-specific antibody, a 95K band was labeled in addition to the 105K species. It is not clear what relationship this species has to the active secreted toxin. At least one of these two forms is located at the cell surface, as judged by indirect immunofluorescence, and is distributed in a nonuniform, punctate pattern. It is possible this punctate distribution reflects structural features of the *P. haemolytica* envelope which are involved in the secretion of the extracellular toxin and may be similar to the zones of adhesion reported by Bayer (3).

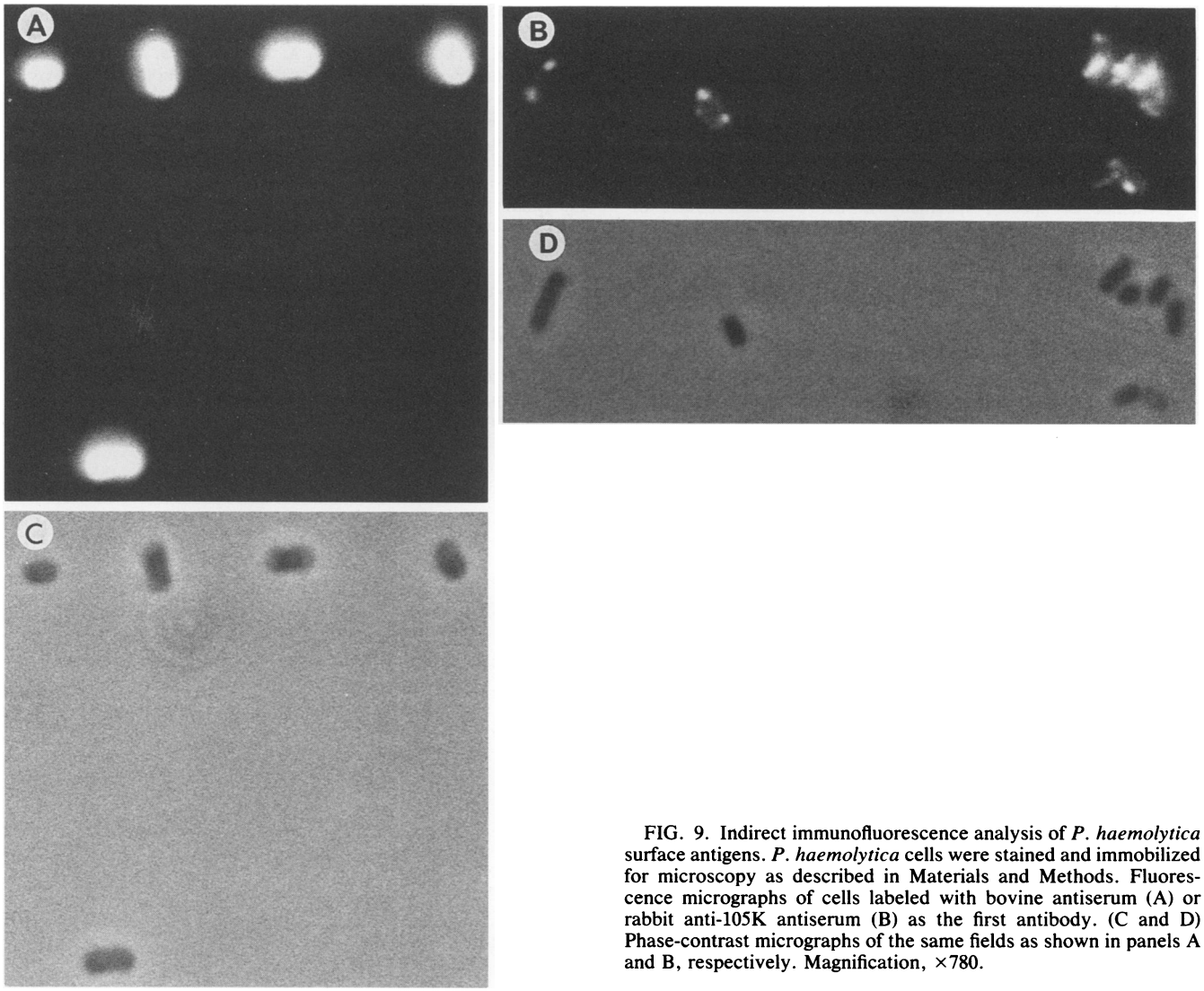

FIG. 9. Indirect immunofluorescence analysis of *P. haemolytica* surface antigens. *P. haemolytica* cells were stained and immobilized for microscopy as described in Materials and Methods. Fluorescence micrographs of cells labeled with bovine antiserum (A) or rabbit anti-105K antiserum (B) as the first antibody. (C and D) Phase-contrast micrographs of the same fields as shown in panels A and B, respectively. Magnification, ×780.

ACKNOWLEDGMENTS

We are grateful to Terri Moulds and Jeff Smith for technical assistance, to Lisa Lohman for her skilled graphics, to Lydia McCartney for administrative assistance, and to the Biocommunications Office of the College of Medicine, Texas A&M University, for photographic services. We are indebted to D. Kraemer and R. Loan of the College of Veterinary Medicine, Texas A&M University, for access to cattle and serum of infected cattle, respectively.

This work was supported by a Texas Advanced Technology Research Program grant to R.Y. and D.S.

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