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Cloning and Expression of the Leukotoxin Gene of *Pasteurella haemolytica* A1 in *Escherichia coli* K-12

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A clone bank of *Pasteurella haemolytica* A1 was constructed by partial digestion of the genomic DNA with *Sau*3A and ligation of 5- to 10-kilobase-pair fragments into the *Bam*HI site of the plasmid vector pBR322. After transformation into *Escherichia coli* K-12, a total of 4×10^3 recombinant clones was obtained. These were screened for the production of *P. haemolytica* soluble antigens by a colony enzyme-linked immunosorbent assay blot method with a rabbit antiserum raised against the soluble antigens. The clones producing *P. haemolytica* soluble antigens were then analyzed for the production of the leukotoxin by a cytotoxicity assay with cells from a bovine leukemia-derived B-lymphocyte cell line as the target cells. Positive clones were identified, and subsequent restriction analysis of the recombinant plasmids showed that the same 6.3 kilobase pairs of insert DNA was cloned in either of the two orientations into the plasmid vector pBR322. One of the clones was selected for further characterization of the leukotoxin as produced in *E. coli*. Tests for heat lability and target cell species specificity with canine, porcine, and human peripheral blood lymphocytes indicated that the activity of the cloned leukotoxin was identical to that of the *P. haemolytica* leukotoxin. Furthermore, the *E. coli*-produced leukotoxin was also neutralized by bovine or rabbit antiserum known to have antitoxic activity. When cellular proteins from the *E. coli* clones were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blot analysis, a 100,000-dalton protein was identified which corresponded to one of the soluble antigens found in the leukotoxic culture supernatant of *P. haemolytica*. These results demonstrated that the gene(s) for the *P. haemolytica* leukotoxin have been cloned and that the leukotoxin was expressed in *E. coli*.

Pneumonic pasteurellosis associated with *Pasteurella haemolytica* serotype 1 is a major cause of economic loss in feedlot cattle (17). Although vaccination with this organism might be expected to produce immunity to the disease, evidence from experimental trials and field studies with conventional formalinized bacterins has failed to show a protective effect (16). In fact, vaccinated animals are frequently more susceptible to the disease than are their nonvaccinated counterparts. However, immunization with live *P. haemolytica* has been shown to protect cattle against experimental challenge exposure to the bacterium (6; C. Smith, Abstr. North Am. Symp. Bov. Resp. Dis., Amarillo, Tex., 1983). Live *P. haemolytica* produces a cytotoxin with specificity for ruminant leukocytes (11, 22). This may contribute to the pathogenesis of pneumonic pasteurellosis by impairing primary lung defense and subsequent immune response or by the induction of inflammation as a consequence of leukocyte lysis. Leukotoxic activity is present in bacteria-free culture supernatant from logarithmic-phase cultures, and in preliminary studies, vaccination of calves with *P. haemolytica* culture supernatant induced resistance to experimental challenge (P. E. Shewen ad B. N. Wilkie, Abstr. Con. of Res. Workers in An. Dis. Chicago, Ill., 1983). However, crude culture supernatant contains other soluble antigens and products in addition to the leukotoxin, including protease, neuraminidase (20), and antigens which induce agglutinating antibody production. To date, the inability to separate these antigens by conventional chemical and physical techniques has hindered understanding of the protective immune response. The importance of various *P. haemolytica* antigens, particularly the leukotoxin, in this response must be clarified to formulate a rational and effective approach to vaccine preparation. Therefore, an alternative method, the isolation of genes coding for the soluble antigens of *P. haemolytica*, was attempted by molecular cloning. In this paper, we report the construction of a plasmid clone bank of the *P. haemolytica* genome and the identification of clones expressing genes for the leukotoxin in *E. coli*.

MATERIALS AND METHODS

Bacteria and plasmid strains. *P. haemolytica* A1 (biotype A, serotype 1) was originally obtained from E. L. Biberstein, University of California, Davis. Stock organisms were maintained as lyophilized cultures after freeze-drying in distilled water containing (wt/vol) 5% dextran (molecular weight, 70,000), 7% sucrose, and 1% monosodium glutamate.

Escherichia coli HB101 was used as the recipient, and plasmid pBR322 was used as the vector for the construction of the clone bank. Their genotypes and characteristics were described by Maniatis et al. (13) and Bolivar et al. (2), respectively.

Media, enzymes, and chemicals. *P. haemolytica* was cultured in brain heart infusion broth (Difco Laboratories, Detroit, Mich.) while *E. coli* cultures were grown in LT medium as previously described (R. Y. C. Lo and L. A. Cameron, submitted for publication). Where appropriate, the antibiotics ampicillin and tetracycline were supplemented at 100 and 12.5 mg/liter, respectively, after the medium was autoclaved. Restriction endonuclease, T4 DNA ligase, *E. coli* DNA polymerase I, and bacterial alkaline phosphate were purchased from Bethesda Research Laboratories, Inc., Gaithersburg, Md.; GIBCO, Burlington, Ontario, Canada; or Boehringer Mannheim, Dorval, Quebec, Canada and used as described by the suppliers. Low-melting-point agarose was from Bethesda Research Labora-

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tories. [α-^{32}P]dATP (3,000 Ci/mmol) was purchased from New England Nuclear Corp., Lachine, Quebec.

Construction of a clone bank of *P. haemolytica* DNA in *E. coli*. A clone bank of *P. haemolytica* A1 was constructed by the method of Nasmyth and Reed (18). Genomic DNA was extracted from *P. haemolytica* A1 by sodium dodecyl sulfate (SDS) lysis of the bacterial cells and phenol extraction (15). The DNA was then partially digested by the restriction endonuclease *Sau*3A, and fragments of 5 to 10 kilobase pairs were recovered by sucrose gradient centrifugation by the method of Maniatis et al. (14). These fragments were inserted into the unique *Bam*HI site of the plasmid vector pBR322 which had been dephosphorylated by bacterial alkaline phosphatase. The ligated DNA mixture was transformed into *E. coli* competent cells, and recombinants were recovered by selecting for ampicillin-resistant (Amp^r) colonies. The recombinants were pooled and amplified in broth cultures, and plasmid DNA was prepared by cesium chloride-ethidium bromide centrifugation (26) and stored at −20°C.

Preparation of rabbit antiserum to soluble antigens and screening of the clone bank. New Zealand White rabbits (Riemens Fur Ranches Ltd., St. Agatha, Ontario, Canada) were immunized with lyophilized *P. haemolytica* culture supernatant prepared essentially as described previously (22) except that 7% autologous rabbit serum was substituted for fetal bovine serum in the growth medium. Each rabbit received a total of seven inoculations of 10 mg of crude supernatant in 1 ml of saline, divided equally intradermally and intravenously, at intervals of 10 to 12 days. The first two intradermal inoculations were emulsified in complete Freund adjuvant (Difco). Serum was collected 1 week after the seventh immunization. This serum had a titer to *P. haemolytica* surface antigens of 1/32, as measured by direct bacterial agglutination (23), and a neutralizing titer to *P. haemolytica* leukotoxin of 1/640, as measured in the microplate assay described below.

The antibody preparation was used to screen the clone bank for production of *P. haemolytica* antigens by the colony enzyme-linked immunosorbent assay (ELISA) blot method (Lo and Cameron, submitted for publication). Briefly, the *E. coli* recombinant clones were grown on nitrocellulose paper, and the cells were lysed with chloroform vapor. The nitrocellulose paper was immersed in a blocking solution of TBS buffer (20 mM Tris hydrochloride [pH 7.5], 500 mM NaCl) containing 3% gelatin, 150 mM NaCl, 5 mM $MgCl_2$, 1 μg of DNase I per ml, and 40 μg of lysozyme per ml for 1 h and then incubated with the rabbit antiserum (1/200 dilution) in TBS buffer overnight. After being washed twice in TBS buffer, the nitrocellulose paper was reacted with goat anti-rabbit immunoglobulin G (IgG) conjugated with horseradish peroxidase (Bio-Rad Laboratories, Mississauga, Ontario, Canada) at a 1/2,000 dilution in TBS buffer for 1 h. The blots were washed twice in TBS buffer and developed in horseradish peroxidase color development reagents (Bio-Rad).

Agarose gel electrophoresis and Southern blot analysis. The restriction fragments of the recombinant plasmids were analyzed by agarose gel electrophoresis as previously described (Lo and Cameron, submitted for publication). For Southern blot analysis (25), *P. haemolytica* genomic DNA was digested with an appropriate restriction endonuclease, electrophoresed on an agarose gel, and blotted onto nitrocellulose paper by electrophoretic transfer. The DNA fragments to be used as probes were recovered from the recombinant plasmids after enzyme digestion and purification on low-melting-point agarose gels by the method of Wieslander (27) and labeled with [^{32}P]dATP by nick-translation by the procedure of Rigby et al. (21).

Preparation of periplasmic and cellular proteins. The *E. coli* cells carrying the recombinant plasmids were subjected to osmotic shock treatment by the method of Neu and Heppel (19). The resulting cell suspension was stirred for 10 min on ice and then centrifuged, and the supernatant was recovered. The supernatant was adjusted to 0.01 M Tris hydrochloride (pH 8), concentrated by centricon units (Amicon Corp., Oakville, Ontario, Canada), stored at 4°C, and designated as the periplasmic protein preparation. The cell pellet was suspended in 10 ml of 0.01 M Tris hydrochloride (pH 8), sonicated at 100 W for 1 min, and centrifuged at 100,000 × *g* for 1 h. The sonicate supernatant recovered was designated as the cellular protein preparation. The enzymes cyclic phosphodiesterase and β-galactosidase were assayed as markers for periplasmic and cellular proteins, respectively, as described previously (19). The protein concentrations of the preparations were determined by the method of Lowry (12).

SDS-polyacrylamide gel electrophoresis and Western blot analysis. The periplasmic and cellular proteins from the *E. coli* recombinant clones were analyzed by SDS-polyacrylamide gel electrophoresis by the method of Hancock and Carey (8). The separating gel consisted of 9% (wt/vol) acrylamide (acrylamide/bisacrylamide ratio, 40:0.8) in 0.4 M Tris hydrochloride (pH 8.8)–0.09 M NaCl–1% SDS, while the stacking gel consisted of 3% (wt/vol) acrylamide (acrylamide/bisacrylamide ratio, 30:0.8) in 0.13 M Tris hydrochloride (pH 7)–1% SDS. For each lane, approximately 15 μg of protein was mixed with the solubilization reduction mixture (8) and electrophoresed at 150 V with 0.025 M Tris–0.2 M glycine (pH 8.4)–1% SDS as the running buffer. Protein bands were visualized by staining with 0.05% Coomassie blue R250 (wt/vol) in 10% acetic acid–14% methanol.

For Western blot analysis (3), the proteins were transferred to nitrocellulose paper after SDS-polyacrylamide gel electrophoresis in a Trans-Blot cell apparatus (Bio-Rad) with 0.025 M Tris–0.2 M glycine (pH 8.4)–20% methanol as the blotting buffer. The *P. haemolytica* proteins were then detected by ELISA with the rabbit antiserum preparation described above.

Evaluation of leukotoxic activity. The leukotoxic activities of periplasmic and cellular protein preparations were measured in a microplate cytotoxicity assay (C. N. Greer and P. E. Shewen, submitted for publication) with BL-3 cells, a bovine leukemia-derived B-lymphocyte cell line (originally obtained from G. Theilen, University of California, Davis), as targets. Cell viability, measured as the uptake of the vital dye neutral red, was determined by reading the optical density of each well at 540 nm with an automated spectrophotometer. After overnight dialysis against RPMI 1640 medium, quadruplicate 200-μl samples (at protein concentrations of 6 mg/ml) were incubated with 2 × 10^5 cell in each of four wells of a microtiter plate for 1 h at 37°C. Lyophilized *P. haemolytica* culture supernatant (22), reconstituted at 3 mg/ml in RPMI 1640 medium, was used as the positive control for toxic activity. Percent toxicity was calculated as the percent loss of viability by comparing the mean optical density in test wells with that in control wells containing cells incubated with RPMI 1640 medium only. The heat lability of toxic activity was determined by preheating an aliquot of each sample at 56°C for 30 min before testing. Host species specificity in toxic activity was confirmed by retest-

ing toxic samples with canine, porcine, and human peripheral blood lymphocytes purified by density gradient centrifugation on Ficoll-Hypaque (24) as targets. In addition, the rabbit antiserum and a bovine serum with antitoxic activity, obtained from an infected calf, were tested in serial twofold dilutions for the ability to neutralize toxicity in one of the clone-derived samples.

RESULTS

Construction of a clone bank of *P. haemolytica*. About 4×10^3 Amp^r *E. coli* colonies were recovered after transformation with the *P. haemolytica* DNA fragments and the pBR322 DNA ligation mixture, of which less than 1% were also tetracycline resistant. The transformants were pooled from the agar plates and amplified in broth cultures in LT medium containing ampicillin, and plasmid DNA was prepared by cesium chloride-ethidium bromide centrifugation for storage (−20°C) as the clone bank. Plasmid DNA of the clone bank was analyzed by agarose gel electrophoresis, which showed that it contained plasmids larger than the vector pBR322 (Fig. 1).

Isolation of recombinant plasmids coding for *P. haemolytica* soluble antigens. Plasmid DNA from the clone bank was transformed into *E. coli* competent cells, and the transformants were screened for *P. haemolytica* antigens by the colony ELISA blot method to detect the production of *P. haemolytica* antigens (Lo and Cameron, submitted for publication). Twenty-seven positive recombinants were identified. Periplasmic and cellular proteins were prepared from the positive clones to assay for the *P. haemolytica* leukotoxin.

Characterization of the recombinant plasmids. Plasmid DNA from the positive recombinant clones was analyzed by restriction endonuclease mapping, and the results indicated that some of the recombinant plasmids had the same insert DNA. Four recombinant plasmids, 10, 11, 16, and 18, were found to have the same restriction map in that a 6.3-kilobase-pair (kbp) insert was cloned in the vector pBR322 (Fig. 2). More interestingly, the same 6.3-kbp insert DNA was also cloned in plasmids 5 and 6 in the opposite orientation (Fig. 2).

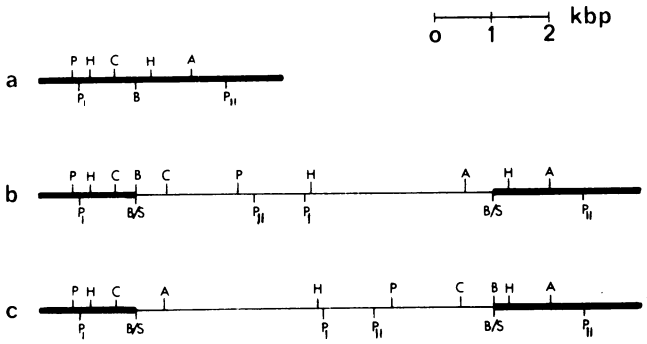

FIG. 2. Restriction maps of the recombinant plasmids which contain the *P. haemolytica* leukotoxin gene. Heavy lines represent pBR322 sequences, and light lines represent insert sequences. (a) Plasmid pBR322 represented linearly at the coordinates of 3 kbp. (b) Recombiant plasmids pPH 5 and pPH 6. (c) Recombinant plasmids pPH 10, pPH 11, pPH 16, and pPH 18. Abbreviations: P, *Pst*I; H, *Hin*cII; C, *Cla*I; A, *Ava*I; PI, *Pvu*I; B, *Bam*HI; PII, *Pvu*II; B/S, *Bam*HI-*Sau*3A junctions. Only one of the *Bam*HI-*Sau*3A junctions regenerated a *Bam*HI site. There are no restriction sites for the following endonucleases on the insert DNA: *Eco*RI, *Bam*HI, *Hin*dIII, *Sal*I, *Nde*I, *Kpn*I, *Sma*I, and *Xba*I.

To demonstrate that the insert DNA was of *P. haemolytica* origin, the *Pst*I-*Ava*I fragment from plasmid 5 was purified, nick-translated with [^{32}P]dATP, and used as a probe in Southern blot analysis against *P. haemolytica* genomic DNA. The results (Fig. 3) indicate that the insert DNA hybridized to unique fragments of the *P. haemolytica* DNA digest.

Testing for the *P. haemolytica* leukotoxin in the *E. coli*

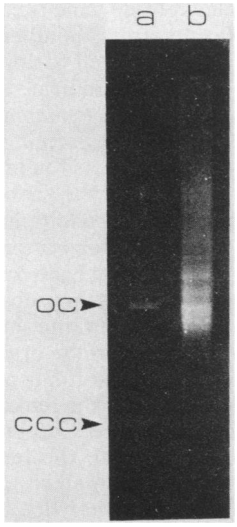

FIG. 1. Agarose gel electrophoresis profile of the *P. haemolytica* plasmid clone bank. Lane a, Plasmid vector pBR322; lane b, plasmid DNA from the *P. haemolytica* clone bank. Shown are covalently closed circular (ccc), and (oc) open circular forms of pBR322.

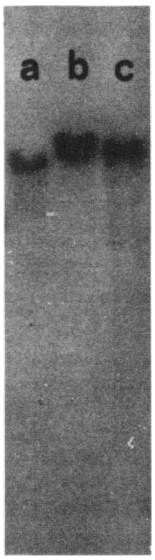

FIG. 3. Southern blot hybridization analysis of insert DNA from pPH 5 against total *P. haemolytica* genomic DNA. The *Pst*I-*Ava*I fragment from pPH 5 was purified, nick-translated with [^{32}P]dATP, and used as a probe in hybridization against *P. haemolytica* genomic DNA digested with *Bam*HI (lane a), *Eco*RI (lane b), and *Hin*dIII (lane c).

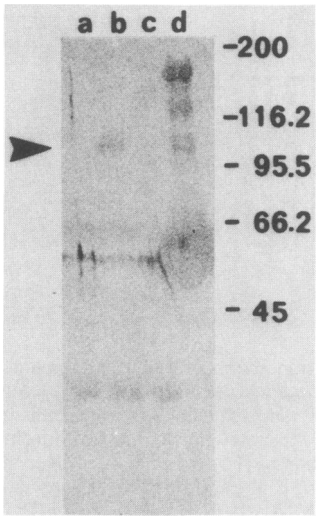

FIG. 4. Western blot analysis of the cellular proteins from the *E. coli* clones carrying pPH 5, pPH 10, and the vector pBR322. Lane a, pPH 10; lane b, pPH 5; lane c, pBR322; lane d, *P. haemolytica* culture supernatant. The location of the additional protein band from the pPH 5 preparation is indicated with an arrowhead. The locations of the molecular weight standards (high-molecular-weight protein standards; Bio-Rad) in thousands are indicated to the right: myosin (200), β-galactosidase (116.2), phosphorylase B (92.5), bovine serum albumin (66.2), and ovalbumin (45).

recombinant clones. Periplasmic and cellular proteins from the *E. coli* clones carrying the recombinant plasmids 1, 5, 8, 9, 10, 11, and 13 as well as plasmid pBR322 were assayed for leukotoxin activity. None of the periplasmic protein preparations showed significant cytotoxic activity. Cellular proteins recovered after sonication from three recombinant clones were found to be toxic for BL-3 cells. These were clones carrying plasmids 5, 10, and 11, which showed 95.4, 53.1, and 55.5% toxicity, respectively. For plasmids 10 and 11, all activity was heat labile (56°C, 1 h), while 26.1% of the toxicity in the plasmid 5 preparation was heat stable. In comparison, the *P. haemolytica* culture supernatant (22) used as a toxicity control was 93.4% toxic and 19.6% toxic after heating.

Cellular proteins from the clone bearing plasmid 5 showed no toxicity for canine, porcine, or human peripheral blood lymphocytes when tested. Likewise, the *P. haemolytica* control was not toxic for these nonruminant cells.

Both bovine and rabbit antitoxic sera neutralized the toxic activity of cellular proteins from the clone bearing plasmid 5 at a 1/256 dilution, the highest dilution tested. No neutralization occurred at any dilution with the normal rabbit serum pool or the fetal calf serum pool, which were used as controls.

SDS-polyacrylamide gel electrophoresis and Western blot analysis. The protein preparations from the recombinant clones carrying plasmid 5 and plasmid 10 were analyzed by SDS-polyacrylamide gel electrophoresis followed by electrophoretic transfer to nitrocellulose paper and ELISA. Results from Coomassie blue staining of the gels indicated no new protein bands in the periplasmic protein preparations. However, an additional protein band was detected after Western blot analysis of the cellular protein preparation from the recombinant clone carrying plasmid 5 (Fig. 4). This additional protein band migrated to a position which corresponds to one of the soluble antigens in the *P. haemolytica* culture supernatant. The molecular weight of the extra protein was estimated to be about 100,000.

In the Western blot analysis (Fig. 4), several other bands were detected in all of the protein preparations. Since these bands were present also in the control sample, *E. coli* carrying pBR322, they are probably *E. coli* proteins which react with the antibody preparation and do not affect the present interpretation.

DISCUSSION

A clone bank of *P. haemolytica* A1 was constructed in the plasmid vector pBR322. Assuming the genomic size of *P. haemolytica* to be similar to that of *E. coli*, 4,100 kbp (10), the number of recovered clones should ensure a 99.7% probability of containing the whole *P. haemolytica* genome according to the equation of Clarke and Carbon (4). When the colony ELISA blot method (Lo and Cameron, submitted for publication) was used as an initial screen of the clone banks, a number of *E. coli* recombinant clones producing *P. haemolytica* soluble antigens were identified.

When the protein preparations from a number of the *E. coli* recombinant clones were assayed for leukotoxic activity, three preparations were found to be toxic for the BL-3 cells. These were the cellular proteins from the clones carrying plasmids 5, 10, and 11. Interestingly, they all carried the same *P. haemolytica* insert DNA in either of the two orientations (Fig. 2). (After the initial toxicity assay, three more recombinant plasmids, 6, 16, and 18, were also identified as carrying the same insert DNA.) These results suggest that the leukotoxin gene is probably carried on the 6.3-kbp insert DNA. Furthermore, the 6.3-kbp DNA must also carry the necessary regulatory regions for the expression of the leukotoxin. At this point, the prefix pPH will be introduced to designate these six plasmids.

The leukotoxic activity of these protein preparations was heat labile, which is characteristic of the *P. haemolytica* leukotoxin. Furthermore, similar to the *P. haemolytica* leukotoxin, the preparation from the recombinant clones carrying pPH 5 was found to be nontoxic for canine, porcine, and human peripheral blood lymphocytes (22). In addition, both rabbit and bovine antitoxic sera neutralized the toxicity in the pPH 5 preparation. Therefore, we are confident that the *P. haemolytica* leukotoxin gene is carried in this 6.3-kbp DNA fragment. Results from Southern blot analysis, in which the insert DNA hybridized to unique DNA fragments of the *P. haemolytica* genome, indicate that there is only one copy of the leukotoxin gene in *P. haemolytica*.

The leukotoxic activity of the protein preparation from the *E. coli* clones carrying pPH 5 was found to be twice those of the protein preparations from the clones carrying pPH 10 and pPH 11. Furthermore, an additional protein band was found in the pPH 5 protein preparation after Western blot analysis (Fig. 4). This additional band was absent in the pPH 10 and the control pBR322 protein preparations. We suggest that there is a higher level of leukotoxin produced when the 6.3-kbp insert DNA is cloned in the orientation seen in pPH 5. This increased level of leukotoxin production may be a result of expression from both the leukotoxin promoter and the tetracycline promoter on pBR322 in pPH 5, while in the other orientation, i.e., pPH 10, the leukotoxin can be expressed only from its own promoter.

The migration of the *P. haemolytica* leukotoxin produced in the *E. coli* recombinant clone carrying pPH 5 during SDS-polyacrylamide gel electrophoresis corresponded to one of the soluble antigens present in the *P. haemolytica* culture supernatant (Fig. 4), suggesting that the molecular

weight of the leukotoxin is about 100,000. Other researchers, by using physical techniques for purification, have reported molecular weights for the *P. haemolytica* leukotoxin of 150,000 (9) and 300,000 (1). These may be overestimated due to the aggregation or binding of serum materials with the leukotoxin. A more accurate determination of the molecular weight of the leukotoxin will be possible after DNA sequence analysis of the cloned gene.

During the initial screening of leukotoxic activity from the recombinant clones, toxic activities were observed only with the cellular protein preparations from *E. coli* containing pPH 5, pPH 10, or pPH 11. No leukotoxic activity was found in the respective periplasmic protein preparations. The failure of the *P. haemolytica* leukotoxin to be excreted by the *E. coli* cells is not unexpected since it has been observed on a number of occasions that foreign proteins produced in *E. coli* from cloned genes were not exported (5, 7).

In conclusion, we have successfully cloned the *P. haemolytica* leukotoxic gene on a 6.3-kbp DNA fragment and obtained expression of leukotoxic activity in *E. coli*. Further investigation and characterization of the cloned gene should provide information on the molecular nature of the leukotoxin as well as on the genetic organization and regulation of expression of the leukotoxin gene. This is important in understanding the pathogenic and immunogenic properties of the leukotoxin and its potential for the production of a vaccine against pneumonic pasteurellosis.

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