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Nucleotide Sequence of the Leukotoxin Genes of *Pasteurella haemolytica* A1

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A 4.4-kilobase-pair DNA fragment coding for the leukotoxin of *Pasteurella haemolytica* A1 has been isolated, and its nucleotide sequence has been determined. Two open reading frames, designated *lktC* and *lktA*, coding for proteins of 19.8 and 101.9 kilodaltons, respectively, were identified. Expression of the two genes in minicell-labeling experiments resulted in the production of the predicted proteins LKTC and LKTA. By using an antiserum against the soluble antigens of *P. haemolytica* A1 in Western blot (immunoblot) analysis of total cellular proteins from the *Escherichia coli* clones, LKTA was identified as an additional antigenic protein. Results from subcloning of the DNA fragment suggested that expression from both *lktC* and *lktA* is required for leukotoxin activity, indicating that the leukotoxin of *P. haemolytica* A1 is encoded by two genes. A comparison of the organization and the DNA sequence of the leukotoxin genes with those of the *E. coli* alpha-hemolysin genes showed a significant degree of homology between the two loci. This analysis suggested that the leukotoxin genes of *P. haemolytica* A1 and the *E. coli* alpha-hemolysin genes may have evolved from a common ancestor and that the two toxins may share similar activities or functional domains or both.

Bovine pneumonic pasteurellosis, also known as shipping fever, is a major cause of sickness and death in the feedlot cattle industry (19, 41). The principal microorganism associated with the disease is *Pasteurella haemolytica* A1. It has been shown that *P. haemolytica* A1 produces a heat-labile cytotoxin which is specific against ruminant leukocytes (14, 33). This leukotoxin has been implicated as a major virulence factor in the pathogenesis of *P. haemolytica* A1 by impairing the primary lung defense mechanism and subsequent immune response or by the induction of inflammation as a consequence of leukocyte lysis. Very little is known about the biochemical nature and the mode of action of the leukotoxin because of the inability to purify it by conventional biochemical techniques. An understanding of the molecular nature of the leukotoxin is important in studying its involvement in the development of the disease. Furthermore, the leukotoxin is a potential candidate for the development of a vaccine against shipping fever, and this would best be accomplished with a full characterization of the molecule.

We recently reported the cloning of the genes coding for the leukotoxin of *P. haemolytica* A1 and its expression in *Escherichia coli* (17). In this paper, we present the nucleotide sequence of the cloned DNA and the identification of the leukotoxin genes. These results provide a basis for further biochemical and genetic analysis of the function and expression of the leukotoxin.

MATERIALS AND METHODS

Bacteria, plasmids, and culture conditions. *E. coli* HB101 and *P. haemolytica* A1 were as described previously (16, 17). *E. coli* TG-1 (F′ *traD36 proAB lacI*^{q} ZΔM15/(*lac pro*) *supE thi-1 hsdD5*) was obtained from Toby Gibson. *E. coli* AT1522 (F^{-} *ara-13 azi-8 tonA2 lacY1 minA1 minB2 rpsL135 xyl-7 mtl-2 thi-1*) was obtained from Eric Vmir. The recombinant plasmid pPH5 coding for the leukotoxin has been described previously (17). The M13 mp18/mp19 vectors were purchased from Pharmacia Chemicals Inc. (Dorval, Quebec, Canada).

The recipe for LT medium and antibiotic supplements was as described (16). The *E. coli* minicell-producing strain AT1522 and strain TG-1, used for the propagation of the M13 phages, were grown in Davis minimal medium (21).

Enzymes and chemicals. All restriction endonucleases and DNA modifying enzymes were purchased from Bethesda Research Laboratories (Burlington, Ontario, Canada) or Pharmacia Chemicals Inc. and used as described by the suppliers. All radioisotopes were purchased from New England Nuclear Corp. (Lachine, Quebec, Canada). The goat anti-rabbit immunoglobulin G-alkaline phosphatase conjugate and the color development reagents were purchased from Bio-Rad Laboratories (Mississauga, Ontario, Canada).

Molecular cloning and DNA sequencing. The dideoxy chain termination method (31) in conjunction with the M13 mp18/mp19 vectors (25, 40) were used to determine the nucleotide sequence of the insert DNA on pPH5 and pPH5A. Before sequencing was done, the insert DNA was subcloned by digestion with the appropriate restriction endonucleases, which generated ends suitable for insertion into the multiple cloning sites of the M13 vectors. Each DNA fragment was purified by low-melting-point agarose gel electrophoresis (39), ligated into the M13 vectors (appropriately digested), and transformed into *E. coli* TG-1 as described previously (16). The protocols for isolation of recombinant single-stranded DNA templates and the dideoxy sequencing reactions were essentially those suggested by Bethesda Research Laboratories with [α-^{32}P]dATP (800 Ci/mol) as the labeling isotope. Electrophoresis was done on 7 M urea–8% polyacrylamide gels by using the sequencing apparatus designed by Tyler Research Corp. (Raleigh, N.C.). To sequence the whole insert DNA, the method of Dale et al. (4) was adopted to generate overlapping deletions of the insert DNA in mp19. Briefly, the primer RD22 (International Biotechnologies, Inc., Toronto, Ontario, Canada) was annealed with the single-stranded DNA template at 65°C. After

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the primer cooled to 40°C, *Eco*RI was added to digest the DNA, generating 3'-OH ends which were susceptible to the 3' to 5' exonuclease activity of T4 DNA polymerase. Digestion was stopped at timed intervals, and the 3'-OH ends were tailed with dGTP by using terminal deoxytransferase. The DNA was reannealed with RD22, ligated, and transformed into TG-1. A set of overlapping deletions was thus generated which permits sequencing of the insert DNA without further subcloning.

DNA sequence analysis. The DNA sequence was analyzed by using the Pustell Sequence Analysis programs (International Biotechnologies, Inc., Toronto, Ontario, Canada) on an IBM PC XT 286 microcomputer (IBM Corp., Armonk, N.Y.). The programs written by Schwindinger and Warner (32) were also used wherever a properly formatted output was required. To aid in studying homology with other published sequences, the GenBank Genetic Sequence Data Bank (Floppy Disk Version Release 44.0) from Bolt, Berank and Newman Laboratories Inc. (Cambridge, Mass.) was used in sequence comparison analyses.

Isolation and radiolabeling of minicells. Minicells were isolated from *E. coli* AT1522 transformed with the different recombinant plasmids. The minicells were purified by sucrose gradient centrifugation from cultures grown in Davis minimal medium supplemented with 0.5% Casamino Acids (Difco Laboratories, Detroit, Mich.) as described by Dougan and Kehoe (5). Isolated minicells were incubated in Davis minimal medium at 37°C for 30 min before the addition of 25 μCi of [^{35}S]methionine, followed by a further incubation of 30 min to label the plasmid-encoded proteins. The minicells were lysed in sodium dodecyl sulfate-polyacrylamide gel electrophoresis sample buffer, and the proteins were separated by polyacrylamide gel electrophoresis using the method of Laemmli (15). Labeled proteins were identified by direct autoradiography of the dried gel by using Du Pont Cronex X-ray film (Du Pont Co., Wilmington, Del.).

Western blot analysis and evaluation of leukotoxic activity. Total cytoplasmic proteins were prepared from *E. coli* clones carrying the different recombinant plasmids for Western blot (immunoblot) analysis as described previously (2). The antiserum used to detect *P. haemolytica* antigens has been described previously (17). This antiserum was further purified by affinity chromatography to remove antibodies which cross-react with *E. coli* proteins in the Western blot analysis (17). Briefly, proteins from *E. coli* HB101 were conjugated to 1 g of preswelled cyanogen bromide-activated Sepharose 4B (Pharmacia Chemicals Inc.) and loaded in a column (1 cm by 10 cm). The antiserum was passed through the column to permit absorption of the cross-reacting antibodies. The gel matrix was then washed with 3 M sodium thiocyanate (pH 7.0) followed by TTBS (20 mM Tris hydrochloride [pH 7.5], 500 mM NaCl, 0.05% Tween 20) to elute any absorbed antibodies and rejuvenate the column (6). Typically, each milliliter of antiserum was passed through the column five times.

The leukotoxic activities of the protein preparations from the *E. coli* clones were analyzed by using the neutral red dye assay as described previously (10).

RESULTS

Isolation of recombinant plasmid pPH5A. Preliminary results on the subcloning of pPH5 (Fig. 1a) showed that no leukotoxic activity could be detected after subcloning of the insert DNA at the *Hin*cII site. The subclones pPH5L and pPH5R (Fig. 1b and c), produced by digestion and religation between the *Hin*cII site and appropriate sites on the vector pBR322, contained the left and right halves of the pPH5 insert DNA. Neither subclone exhibits any toxic activity (Table 1), suggesting that the *Hin*cII site is located in a region required for leukotoxin production. Subsequent DNA sequencing of this region indicated the presence of two open reading frames (ORFs); these are designated ORF1 and ORF2 (Fig. 1), with the *Hin*cII site situated within ORF2. ORF1 was found to be incomplete in that it lacked a termination codon and the protein encoded by this region would have an altered carboxy terminus. Therefore, it became necessary to isolate DNA sequences further to the left of the *Bam*HI site on pPH5 to complete ORF1.

To isolate these sequences, the 1.7-kilobase-pair (kbp) *Bam*HI-*Pst*I fragment from pPH5 (Fig. 1a) was purified by low-melting-point agarose electrophoresis, labeled with [α-^{32}P]dATP by nick translation (29), and used as a probe to screen the *P. haemolytica* A1 clone bank. About 2×10^3 *E. coli* clones were filtered onto two hydrophobic membrane grid filters as described previously (16). A copy of the colonies was made on nitrocellulose paper, and the colonies were lysed and probed with the above fragment by using the colony blot method of Grunstein and Hogness (11). Positive clones were recovered, and the recombinant plasmids were analyzed by restriction endonuclease digestion. A plasmid which contained insert DNA overlapping to the left of the *Bam*HI site on pPH5 was identified and designated pPH5A (Fig. 1d). The nucleotide sequence of the insert DNA was then determined as described above.

Subcloning of the leukotoxin genes. The results of the subcloning and DNA sequencing analysis of the insert DNA on pPH5 and pPH5A are shown in Fig. 1; the two ORFs are bounded by the *Eco*RV and *Xba*I sites of pPH5 and pPH5A, respectively. The initial subcloning step involved the isolation of the 3.1-kbp *Eco*RV-*Eco*RV fragment from pPH5 and its ligation into the *Eco*RV site of pPH5A to replace the *Eco*RV-*Pvu*I region. Plasmid pLKT4 was thus constructed with the insert DNA shown, as confirmed by restriction endonuclease analysis (Fig. 1e). This plasmid represented a fusion of the coding regions of pPH5 and pPH5A such that both ORFs are complete. The sequences to the left of the *Xba*I site on pLKT4 were removed through fusion with the *Sal*I site on the pBR322 vector to form pLKT5 (Fig. 1f). This final subclone was found to express leukotoxin activity when tested in the neutral red dye assay (Table 1), demonstrating that the leukotoxin-coding regions are contained within the insert DNA.

Minicell labeling and Western blot analysis of the cloned leukotoxin. Plasmid pLKT5 was transformed into the *E. coli* minicell-producing strain AT1522 and the plasmid-encoded proteins were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis after being labeled with [^{35}S]methionine. The results presented in Fig. 2 showed that under mild denaturing conditions (65°C; 2 min), two extra proteins were labeled, with molecular sizes of 20 and 100 kilodaltons (kDa). It was observed further that the 100-kDa protein was unstable on being heated at 100°C (results not shown).

Total cytoplasmic proteins were also prepared from *E. coli* HB101 transformed with pLKT5 for Western blot analysis. The results shown in Fig. 3 showed an extra antigenic protein with a molecular size of 100 kDa expressed from pLKT5. This 100-kDa protein comigrates with one of the soluble antigens of *P. haemolytica* A1 and also corresponds to the 100-kDa protein detected in the minicell-labeling experiments. No other antigenic proteins, including the

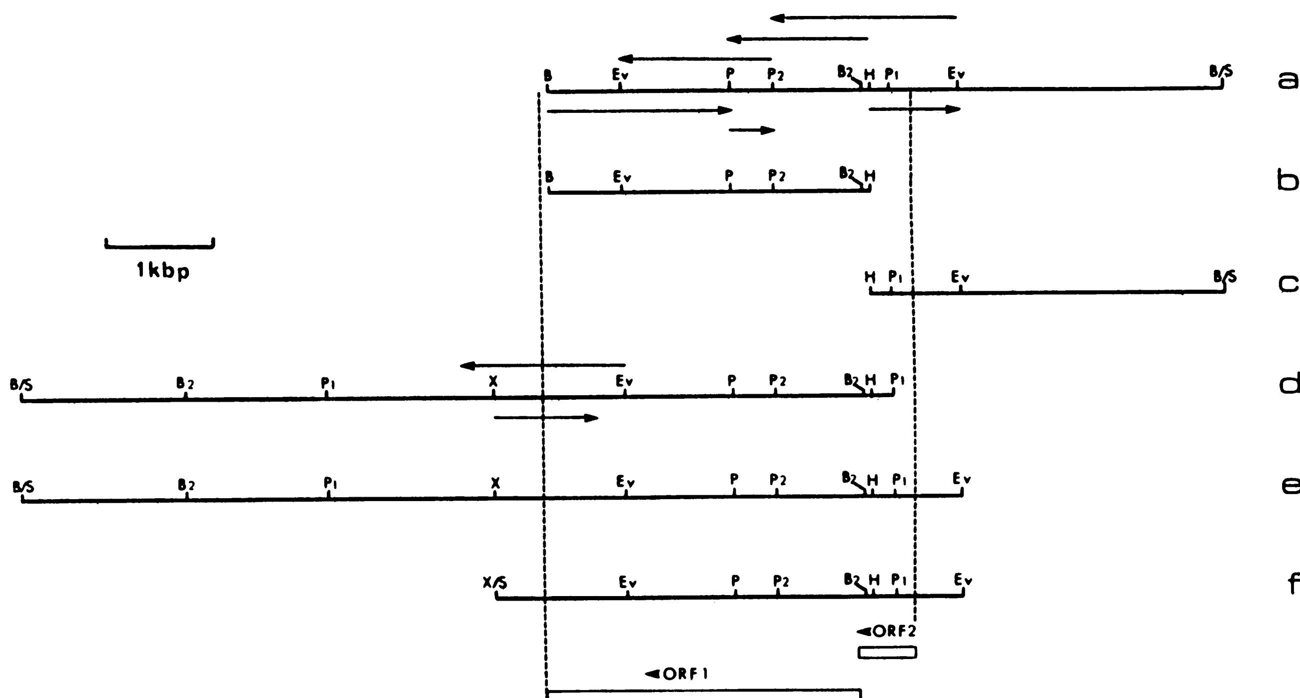

FIG. 1. Restriction maps of the insert DNA fragments carried on the various plasmids used in this study: a, pPH5; b, pPH5L; c, pPH5R; d, pPH5A; e, pLKT4; f, pLKT5. Shown also in parts a and d is the sequencing strategy used to determine the nucleotide sequence of the leukotoxin-coding region; the arrows indicate the direction and extent of DNA sequenced for each fragment. The orientation of the two ORFs is shown below the restriction maps; the vertical dotted lines indicate the extent of the coding regions with respect to each of the insert DNA fragments. The *Bam*HI site shown in parts a and b is regenerated between the insert DNA and the vector pBR322 sequence and therefore is not present on the other insert DNAs. Abbreviations: B, *Bam*HI; Ev, *Eco*RV; P, *Pst*I; H, *Hin*cII; P_1, *Pvu*I; P_2, *Pvu*II; X, *Xba*I; B_2, *Bgl*II; B/S, *Bam*HI-*Sau*3A junction; X/S, *Xba*I-*Sal*I junction.

20-kDa protein observed in the minicell-labeling experiment, were detected in this analysis.

DNA sequence of the leukotoxin genes. The nucleotide sequence of the insert DNA on pLKT5 is presented in Fig. 4. This sequence covered a continuous region of 4,394 bp. Seventy-five percent of both DNA strands was sequenced from the subclones either directly or by the use of overlapping deletions into the cloned DNA. In all cases, each nucleotide was sequenced at least three times independently by using different clones produced by the overlapping deletions of the insert DNA fragments on the M13 mp19 vector.

On analysis of the nucleotide sequence of the insert DNA on pLKT5 by using the Pustell Sequence Analysis programs, two ORFs were identified as shown in the orientations in Fig. 1. The sequence is arbitrarily numbered starting at −469 (at the *Eco*RV site) to correspond to the orientation and location of the ORFs. ORF2 spanned 498 nucleotides, coding for a polypeptide of 166 amino acids with a molecular size of 19.8 kDa, whereas ORF1 spanned 2,856 nucleotides, coding for a polypeptide of 953 amino acids with a molecular size of 101.9 kDa. These two polypeptides coincide in size with the 20- and 100-kDa proteins detected in the minicell-labeling experiments, suggesting that the two ORFs represent the genes coding for the leukotoxin. These genes are designated *lktC* and *lktA*.

The nucleotide sequences upstream from *lktC* exhibit features similar to the promoter sequences commonly found in *E. coli* (Fig. 5a). Three sequences which resembled the TATAAT consensus promoter sequence (12, 30) were identified. Further upstream were sequences similar to the consensus RNA polymerase-binding site, TTGACA. In addition to these potential promoter sequences, a ribosome-binding site was also found preceding the initiation codon of *lktC*. The deduced RNA sequence of this site corresponded very closely to that of the *E. coli* consensus sequence of AAG GAGGU (34). Similar to *lktC*, potential sequences for transcriptional start and ribosome binding were also detected upstream of the initiation codon of *lktA* (Fig. 5b). It is likely that some of these features are involved in the expression of the two genes.

With respect to termination of transcription, a mRNA structure very similar to the rho-independent transcriptional

TABLE 1. Leukotoxic activity of cellular proteins

Sample	% Toxicity[a]	
	Unheated	Heated (56°C, 1 h)
E. coli plasmid		
pBR322	—[b]	—
pPH5	53.8	17.6
pPH5L	—	—
pPH5R	—	—
pLKT5	73.6	30.8
P. haemolytica culture supernatant	83.2	18.7

[a] Percent toxicity as measured by the uptake of the vital dye neutral red by BL3 cells; average of quadruplicate measurements.

[b] —, No significant dye uptake compared with BL3 cells incubated in RPMI 1640 only; $P > 0.1$ by Student's *t* test.

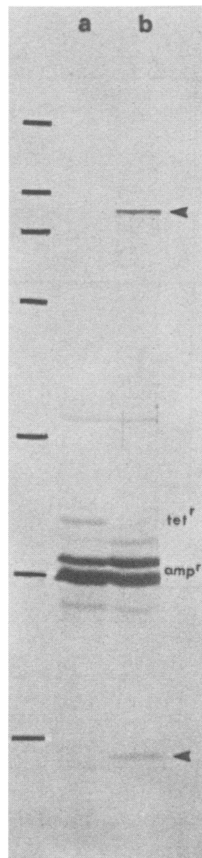

FIG. 2. Minicell labeling of plasmid-encoded proteins. Autoradiogram of a 10% sodium dodecyl sulfate-polyacrylamide gel containing [^{35}S]methionine-labeled proteins expressed from (a) *E. coli*(pBR322) and (b) *E. coli*(pLKT5). The arrows indicate two proteins of 100 and 20 kDa which are expressed from the leukotoxin genes on pLKT5. Shown also are the plasmid-encoded Amp^r and Tet^r proteins expressed from pBR322. Note that pLKT5 confers a Tet^s phenotype due to insertional inactivation and loss of the corresponding protein. The molecular-size standards shown on the left are 200, 116, 92.5, 66, 45, 31, and 21 kDa, from top to bottom.

termination signals of *E. coli* (30) could be identified downstream of *lktA*. This structure consisted of a 16-bp stem and loop region followed by eight uridylate residues of nine nucleotides (Fig. 6). No other such structures were found elsewhere, including in the region following *lktC*.

Leukotoxin genes and *E. coli* alpha-hemolysin genes. When the genetic maps of the leukotoxin genes and the *E. coli* alpha-hemolysin genes were compared, it could be seen that the genetic organization of the two loci was very similar (Fig. 7a). Both loci contain a short coding region followed by a much longer coding region; the regions are of almost identical lengths for each of the loci. The nucleotide sequence of the alpha-hemolysin genes was retrieved from the GenBank data base, and its homology with the leukotoxin genes was analyzed by using the Pustell Sequence Analysis programs (27, 28). Extensive homology was found over the entire coding regions between *lktC* and *hlyC*; similarly, *lktA* and *hlyA* also showed many regions of homology (Fig. 7b). In addition, the matrix showed sequence duplication near the carboxy terminus of the *lktA* and the *hlyA* genes. No homology was detected when the leukotoxin sequence was compared with the sequences of other reported bacterial toxins.

Further comparison between the amino acid sequences of the corresponding polypeptides (LKTC versus HLYC and LKTA versus HLYA) showed even more striking similarities (C. A. Strathdee and R. Y. C. Lo, manuscript in preparation). Briefly, LKTC and HLYC are 166 and 169 amino acids long, respectively, with a 50.3% identical match of the amino acids over the entire polypeptides. On the other hand, LKTA and HLYA are 953 and 1,023 amino acids long, respectively, with a 36.4% identical match in the overall amino acids. Furthermore, LKTA contains a series of repeating amino acid domains near the carboxy terminus, which are essentially identical to the series of repeating amino acid domains identified in HLYA (38), perhaps indicating that these domains are involved in a key common function of the two toxins. These similarities suggest that the *P. haemolytica* A1 leukotoxin and the *E. coli* alpha-hemolysin may have evolved from a common ancestor or share common functional domains (or both) and that the two

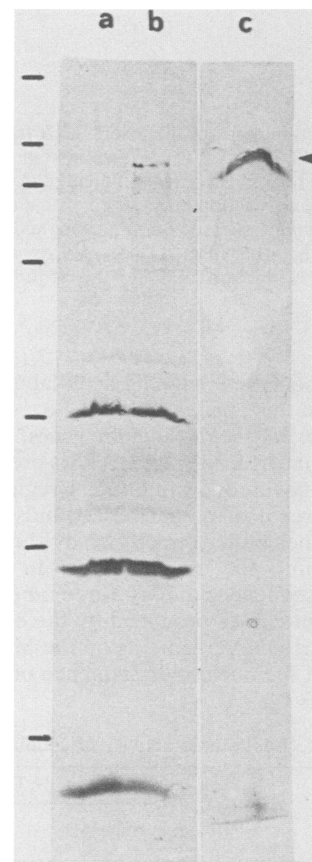

FIG. 3. Western blot analysis of the plasmid-encoded proteins from the *E. coli* clones. Shown here is a blot of a 10% sodium dodecyl sulfate-polyacrylamide gel probed with antiserum specific for *P. haemolytica* A1 culture supernatant antigens. Lanes: a, total cellular proteins from *E. coli*(pBR322); b, total cellular proteins from *E. coli*(pLKT5); c, culture supernatant from *P. haemolytica* A1. The arrowhead indicates the additional antigenic protein present in the pLKT5 preparation which comigrates with the leukotoxin control at 100 kDa. The other antigenic proteins are cross-reacting materials present in both the pLKT5 and pBR322 preparations. The molecular-size standards shown on the left are the same as those described in the legend to Fig. 2.

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-469       -460       -450       -440       -430       -420
GATATCTTG  TGCCTGCGCA GTAACCACAC ACCCGAATAA AAGGGTCAAA AGTGTTTTTT
  E

-410       -400       -390       -380       -370       -360
TCATAAAAAG TCCCTGTGTT TTCATTATAA GGATTACCAC TTTAACGCAG TTACTTTCTT

-350       -340       -330       -320       -310       -300
AAAAAAAGTC TTCTTTTCAT AAAGTTTGTT TTATGTCATA CAAACACATC AAATTGAGAT

-290       -280       -270       -260       -250       -240
GTAGTTTCTC AATCCTCTTG ATTCCTCTAT CTCAAAAAAA CAACCCAAAA GAAAAAAGAA

-230       -220       -210       -200       -190       -180
AAGTATATGT TACATTAATA TTACAATGTA ATTATTTTGT TTAATTTCCC TACATTTTGT

-170       -160       -150       -140       -130       -120
ATAACTTTAA AACACTCCTT TTTCTCTTCT GATTATATAA AAGACAAAAA ATACAATTTA

-110       -100       -90        -80        -70        -60
AGCTACAAAA AACAACAAAA AACAACAAAA AACACGACAA TAAGATCGAG TAATGATTAT

-50        -40        -30        -20        -10
ATTATGTTAT AATTTTTGAC CTAATTTAGA ATAATTATCG AGTGCAAATT ATG AAT CAA
                                                      Met Asn Gln

    15              30              45              60
TCT TAT TTT AAC TTA CTA GGA AAC ATT ACT TGG CTA TGG ATG AAC TCC TCC CTC
Ser Tyr Phe Asn Leu Leu Gly Asn Ile Thr Trp Leu Trp Met Asn Ser Ser Leu

            75              90              105
CAC AAA GAA TGG AGC TGT GAA CTA CTA GCA CGC AAT GTG ATT CCT GCA ATT GAA
His Lys Glu Trp Ser Cys Glu Leu Leu Ala Arg Asn Val Ile Pro Ala Ile Glu

120             135             150             165
AAT GAA CAA TAT ATG CTA CTT ATA GAT AAC GGT ATT CCG ATC GCT TAT TGT AGT
Asn Glu Gln Tyr Met Leu Leu Ile Asp Asn Gly Ile Pro Ile Ala Tyr Cys Ser

    180             195             210             225
TGG GCA GAT TTA AAC CTT GAG ACT GAG GTG AAA TAT ATT AAG GAT ATT AAT TCG
Trp Ala Asp Leu Asn Leu Glu Thr Glu Val Lys Tyr Ile Lys Asp Ile Asn Ser

            240             255             270
TTA ACA CCA GAA GAA TGG CAG TCT GGT GAC AGA CGC TGG ATT ATT GAT TGG GTA
Leu Thr Pro Glu Glu Trp Gln Ser Gly Asp Arg Arg Trp Ile Ile Asp Trp Val

    285             300             315             330
GCA CCA TTC GGA CAT TCT CAA TTA CTT TAT AAA AAA ATG TGT CAG AAA TAC CCT
Ala Pro Phe Gly His Ser Gln Leu Leu Tyr Lys Lys Met Cys Gln Lys Tyr Pro

            345             360             375
GAT ATG ATC GTC AGA TCT ATA CGC TTT TAT CCA AAG CAG AAA GAA TTA GGC AAA
Asp Met Ile Val Arg Ser Ile Arg Phe Tyr Pro Lys Gln Lys Glu Leu Gly Lys

390             405             420             435
ATT GCC TAC TTT AAA GGA GGT AAA TTA GAT AAA AAA ACA GCA AAA AAA CGT TTT
Ile Ala Tyr Phe Lys Gly Gly Lys Leu Asp Lys Lys Thr Ala Lys Lys Arg Phe

    450             465             480             495
GAT ACA TAT CAA GAA GAG CTG GCA ACA GCA CTT AAA AAT GAA TTT AAT TTT ATT
Asp Thr Tyr Gln Glu Glu Leu Ala Thr Ala Leu Lys Asn Glu Phe Asn Phe Ile

            510       519     525             540
AAA AAA TAG AAGGAG ACATCCCTT ATG GGA ACT AGA CTT ACA ACC CTA TCA AAT
Lys Lys  *                   Met Gly Thr Arg Leu Thr Thr Leu Ser Asn

    555             570             585             600
GGG CTA AAA AAC ACT TTA ACG GCA ACC AAA AGT GGC TTA CAT AAA GCC GGT CAA
Gly Leu Lys Asn Thr Leu Thr Ala Thr Lys Ser Gly Leu His Lys Ala Gly Gln

            615             630             645
TCA TTA ACC CAA GCC GGC AGT TCT TTA AAA ACT GGG GCA AAA AAA ATT ATC CTC
Ser Leu Thr Gln Ala Gly Ser Ser Leu Lys Thr Gly Ala Lys Lys Ile Ile Leu

660             675             690             705
TAT ATT CCC CAA AAT TAC CAA TAT GAT ACT GAA CAA GGT AAT GGT TTA CAG GAT
Tyr Ile Pro Gln Asn Tyr Gln Tyr Asp Thr Glu Gln Gly Asn Gly Leu Gln Asp

    720             735             750             765
TTA GTC AAA GCG GCC GAA GAG TTG GGG ATT GAG GTA CAA AGA GAA GAA CGC AAT
Leu Val Lys Ala Ala Glu Glu Leu Gly Ile Glu Val Gln Arg Glu Glu Arg Asn

            780             795             810
AAT ATT GCA ACA GCT CAA ACC AGT TTA GGC ACG ATT CAA ACC GCT ATT GGC TTA
Asn Ile Ala Thr Ala Gln Thr Ser Leu Gly Thr Ile Gln Thr Ala Ile Gly Leu

    825             840             855             870
ACT GAG CGT GGC ATT GTG TTA TCC GCT CCA CAA ATT GAT AAA TTG CTA CAG AAA
Thr Glu Arg Gly Ile Val Leu Ser Ala Pro Gln Ile Asp Lys Leu Leu Gln Lys

            885             900             915
ACT AAA GCA GGC CAA GCA TTA GGT TCT GCC GAA AGC ATT GTA CAA AAT GCA AAT
Thr Lys Ala Gly Gln Ala Leu Gly Ser Ala Glu Ser Ile Val Gln Asn Ala Asn

930             945             960             975
AAA GCC AAA ACT GTA TTA TCT GGC ATT CAA TCT ATT TTA GGC TCA GTA TTG GCT
Lys Ala Lys Thr Val Leu Ser Gly Ile Gln Ser Ile Leu Gly Ser Val Leu Ala

    990             1005            1020            1035
GGA ATG GAT TTA GAT GAG GCC TTA CAG AAT AAC AGC AAC CAA CAT GCT CTT GCT
Gly Met Asp Leu Asp Glu Ala Leu Gln Asn Asn Ser Asn Gln His Ala Leu Ala

            1050            1065            1080
AAA GCT GGC TTG GAG CTA ACA AAT TCA TTA ATT GAA AAT ATT GCT AAT TCA GTA
Lys Ala Gly Leu Glu Leu Thr Asn Ser Leu Ile Glu Asn Ile Ala Asn Ser Val
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FIG. 4. Nucleotide sequence of the insert DNA on pLKT5. The numbers above each line refer to the nucleotide position, which is arbitrarily numbered from −469 at the *Eco*RV site to 3,920 at the *Xba*I site. The predicted amino acid sequences for LKTC and LKTA are shown beneath the DNA sequences. Abbreviations: E, *Eco*RV; X, *Xba*I.

proteins are probably antigenically related. Preliminary data obtained by using an antiserum raised against the alpha-hemolysin (provided by R. Welch) indeed showed immunological cross-reactivity with the *P. haemolytica* leukotoxin in Western blot analysis (manuscript in preparation).

DISCUSSION

Results of the leukotoxic assay of the *E. coli* clones carrying pLKT5 showed that the leukotoxin is carried on the 4.4-kbp DNA on pLKT5. DNA sequence analysis of this

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1095                1110                1125                1140
AAA ACA CTT GAC GAA TTT GGT GAG CAA ATT AGT CAA TTT GGT TCA AAA CTA CAA
Lys Thr Leu Asp Glu Phe Gly Glu Gln Ile Ser Gln Phe Gly Ser Lys Leu Gln

        1155                1170                1185
AAT ATC AAA GGC TTA GGG ACT TTA GGA GAC AAA CTC AAA AAT ATC GGT GGA CTT
Asn Ile Lys Gly Leu Gly Thr Leu Gly Asp Lys Leu Lys Asn Ile Gly Gly Leu

1200                1215                1230                1245
GAT AAA GCT GGC CTT GGT TTA GAT GTT ATC TCA GGG CTA TTA TCG GGC GCA ACA
Asp Lys Ala Gly Leu Gly Leu Asp Val Ile Ser Gly Leu Leu Ser Gly Ala Thr

    1260                1275                1290                1305
GCT GCA CTT GTA CTT GCA GAT AAA AAT GCT TCA ACA GCT AAA AAA GTG GGT GCG
Ala Ala Leu Val Leu Ala Asp Lys Asn Ala Ser Thr Ala Lys Lys Val Gly Ala

        1320                1335                1350
GGT TTT GAA TTG GCA AAC CAA GTT GTT GGT AAT ATT ACC AAA GCC GTT TCT TCT
Gly Phe Glu Leu Ala Asn Gln Val Val Gly Asn Ile Thr Lys Ala Val Ser Ser

1365                1380                1395                1410
TAC ATT TTA GCC CAA CGT GTT GCA GCA GGT TTA TCT TCA ACT GGG CCT GTG GCT
Tyr Ile Leu Ala Gln Arg Val Ala Ala Gly Leu Ser Ser Thr Gly Pro Val Ala

        1425                1440                1455
GCT TTA ATT GCT TCT ACT GTT TCT CTT GCG ATT AGC CCA TTA GCA TTT GCC GGT
Ala Leu Ile Ala Ser Thr Val Ser Leu Ala Ile Ser Pro Leu Ala Phe Ala Gly

1470                1485                1500                1515
ATT GCC GAT AAA TTT AAT CAT GCA AAA AGT TTA GAG AGT TAT GCC GAA CGC TTT
Ile Ala Asp Lys Phe Asn His Ala Lys Ser Leu Glu Ser Tyr Ala Glu Arg Phe

    1530                1545                1560                1575
AAA AAA TTA GGC TAT GAC GGA GAT AAT TTA TTA GCA GAA TAT CAG CGG GGA ACA
Lys Lys Leu Gly Tyr Asp Gly Asp Asn Leu Leu Ala Glu Tyr Gln Arg Gly Thr

        1590                1605                1620
GGG ACT ATT GAT GCA TCG GTT ACT GCA ATT AAT ACC GCA TTG GCC GCT ATT GCT
Gly Thr Ile Asp Ala Ser Val Thr Ala Ile Asn Thr Ala Leu Ala Ala Ile Ala

1635                1650                1665                1680
GGT GGT GTG TCT GCT GCT GCA GCC GGC TCG GTT ATT GCT TCA CCG ATT GCC TTA
Gly Gly Val Ser Ala Ala Ala Ala Gly Ser Val Ile Ala Ser Pro Ile Ala Leu

        1695                1710                1725
TTA GTA TCT GGG ATT ACC GGT GTA ATT TCT ACG ATT CTG CAA TAT TCT AAA CAA
Leu Val Ser Gly Ile Thr Gly Val Ile Ser Thr Ile Leu Gln Tyr Ser Lys Gln

1740                1755                1770                1785
GCA ATG TTT GAG CAC GTT GCA AAT AAA ATT CAT AAC AAA ATT GTA GAA TGG GAA
Ala Met Phe Glu His Val Ala Asn Lys Ile His Asn Lys Ile Val Glu Trp Glu

    1800                1815                1830                1845
AAA AAT AAT CAC GGT AAG AAC TAC TTT GAA AAT GGT TAC GAT GCC CGT TAT CTT
Lys Asn Asn His Gly Lys Asn Tyr Phe Glu Asn Gly Tyr Asp Ala Arg Tyr Leu

        1860                1875                1890
GCG AAT TTA CAA GAT AAT ATG AAA TTC TTA CTG AAC TTA AAC AAA GAG TTA CAG
Ala Asn Leu Gln Asp Asn Met Lys Phe Leu Leu Asn Leu Asn Lys Glu Leu Gln

1905                1920                1935                1950
GCA GAA CGT GTC ATC GCT ATT ACT CAG CAG CAA TGG GAT AAC AAC ATT GGT GAT
Ala Glu Arg Val Ile Ala Ile Thr Gln Gln Gln Trp Asp Asn Asn Ile Gly Asp

        1965                1980                1995
TTA GCT GGT ATT AGC CGT TTA GGT GAA AAA GTC CTT AGT GGT AAA GCC TAT GTG
Leu Ala Gly Ile Ser Arg Leu Gly Glu Lys Val Leu Ser Gly Lys Ala Tyr Val

2010                2025                2040                2055
GAT GCG TTT GAA GAA GGC AAA CAC ATT AAA GCC GAT AAA TTA GTA CAG TTG GAT
Asp Ala Phe Glu Glu Gly Lys His Ile Lys Ala Asp Lys Leu Val Gln Leu Asp

    2070                2085                2100                2115
TCG GCA AAC GGT ATT ATT GAT GTG AGT AAT TCG GGT AAA GCG AAA ACT CAG CAT
Ser Ala Asn Gly Ile Ile Asp Val Ser Asn Ser Gly Lys Ala Lys Thr Gln His

        2130                2145                2160
ATC TTA TTC AGA ACG CCA TTA TTG ACG CCG GGA ACA GAG CAT CGT GAA CGC GTA
Ile Leu Phe Arg Thr Pro Leu Leu Thr Pro Gly Thr Glu His Arg Glu Arg Val

    2175                2190                2205                2220
CAA ACA GGT AAA TAT GAA TAT ATT ACC AAG CTC AAT ATT AAC CGT GTA GAT AGC
Gln Thr Gly Lys Tyr Glu Tyr Ile Thr Lys Leu Asn Ile Asn Arg Val Asp Ser

        2235                2250                2265
TGG AAA ATT ACA GAT GGT GCA GCA AGT TCT ACC TTT GAT TTA ACT AAC GTT GTT
Trp Lys Ile Thr Asp Gly Ala Ala Ser Ser Thr Phe Asp Leu Thr Asn Val Val

2280                2295                2310                2325
CAG CGT ATT GGT ATT GAA TTA GAC AAT GCT GGA AAT GTA ACT AAA ACC AAA GAA
Gln Arg Ile Gly Ile Glu Leu Asp Asn Ala Gly Asn Val Thr Lys Thr Lys Glu

    2340                2355                2370                2385
ACA AAA ATT ATT GCC AAA CTT GGT GAA GGT GAT GAC AAC GTA TTT GTT GGT TCT
Thr Lys Ile Ile Ala Lys Leu Gly Glu Gly Asp Asp Asn Val Phe Val Gly Ser

        2400                2415                2430
GGT ACG ACG GAA ATT GAT GGC GGT GAA GGT TAC GAC CGA GTT CAC TAT AGC CGT
Gly Thr Thr Glu Ile Asp Gly Gly Glu Gly Tyr Asp Arg Val His Tyr Ser Arg

    2445                2460                2475                2490
GGA AAC TAT GGT GCT TTA ACT ATT GAT GCA ACC AAA GAG ACC GAG CAA GGT AGT
Gly Asn Tyr Gly Ala Leu Thr Ile Asp Ala Thr Lys Glu Thr Glu Gln Gly Ser

        2505                2520                2535
TAT ACC GTA AAT CGT TTC GTA GAA ACC GGT AAA GCA CTA CAC GAA GTG ACT TCA
Tyr Thr Val Asn Arg Phe Val Glu Thr Gly Lys Ala Leu His Glu Val Thr Ser

2550                2565                2580                2595
ACC CAT ACC GCA TTA GTG GGC AAC CGT GAA GAA AAA ATA GAA TAT CGT CAT AGC
Thr His Thr Ala Leu Val Gly Asn Arg Glu Glu Lys Ile Glu Tyr Arg His Ser
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FIG. 4. (*Continued*)

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    2610          2625          2640          2655
AAT AAC CAG CAC CAT GCC GGT TAT TAC ACC AAA GAT ACC TTG AAA GCT GTT GAA
Asn Asn Gln His His Ala Gly Tyr Tyr Thr Lys Asp Thr Leu Lys Ala Val Glu

        2670          2685          2700
GAA ATT ATC GGT ACA TCA CAT AAC GAT ATC TTT AAA GGT AGT AAG TTC AAT GAT
Glu Ile Ile Gly Thr Ser His Asn Asp Ile Phe Lys Gly Ser Lys Phe Asn Asp

 2715          2730          2745          2760
GCC TTT AAC GGT GGT GAT GGT GTC GAT ACT ATT GAC GGT AAC GAC GGC AAT GAC
Ala Phe Asn Gly Gly Asp Gly Val Asp Thr Ile Asp Gly Asn Asp Gly Asn Asp

     2775          2790          2805
CGC TTA TTT GGT GGT AAA GGC GAT GAT ATT CTC GAT GGT GGA AAT GGT GAT GAT
Arg Leu Phe Gly Gly Lys Gly Asp Asp Ile Leu Asp Gly Gly Asn Gly Asp Asp

2820          2835          2850          2865
TTT ATC GAT GGC GGT AAA GGC AAC GAC CTA TTA CAC GGT GGC AAG GGC GAT GAT
Phe Ile Asp Gly Gly Lys Gly Asn Asp Leu Leu His Gly Gly Lys Gly Asp Asp

    2880          2895          2910          2925
ATT TTC GTT CAC CGT AAA GGC GAT GGT AAT GAT ATT ATT ACC GAT TCT GAC GGC
Ile Phe Val His Arg Lys Gly Asp Gly Asn Asp Ile Ile Thr Asp Ser Asp Gly

        2940          2955          2970
AAT GAT AAA TTA TCA TTC TCT GAT TCG AAC TTA AAA GAT TTA ACA TTT GAA AAA
Asn Asp Lys Leu Ser Phe Ser Asp Ser Asn Leu Lys Asp Leu Thr Phe Glu Lys

 2985          3000          3015          3030
GTT AAA CAT AAT CTT GTC ATC ACG AAT AGC AAA AAA GAG AAA GTG ACC ATT CAA
Val Lys His Asn Leu Val Ile Thr Asn Ser Lys Lys Glu Lys Val Thr Ile Gln

     3045          3060          3075
AAC TGG TTC CGA GAG GCT GAT TTT GCT AAA GAA GTG CCT AAT TAT AAA GCA ACT
Asn Trp Phe Arg Glu Ala Asp Phe Ala Lys Glu Val Pro Asn Tyr Lys Ala Thr

3090          3105          3120          3135
AAA GAT GAG AAA ATC GAA GAA ATC ATC GGT CAA AAT GGC GAG CGG ATC ACC TCA
Lys Asp Glu Lys Ile Glu Glu Ile Ile Gly Gln Asn Gly Glu Arg Ile Thr Ser

    3150          3165          3180          3195
AAG CAA GTT GAT GAT CTT ATC GCA AAA GGT AAC GGC AAA ATT ACC CAA GAT GAG
Lys Gln Val Asp Asp Leu Ile Ala Lys Gly Asn Gly Lys Ile Thr Gln Asp Glu

        3210          3225          3240
CTA TCA AAA GTT GTT GAT AAC TAT GAA TTG CTC AAA CAT AGC AAA AAT GTG ACA
Leu Ser Lys Val Val Asp Asn Tyr Glu Leu Leu Lys His Ser Lys Asn Val Thr

 3255          3270          3285          3300
AAC AGC TTA GAT AAG TTA ATC TCA TCT GTA AGT GCA TTT ACC TCG TCT AAT GAT
Asn Ser Leu Asp Lys Leu Ile Ser Ser Val Ser Ala Phe Thr Ser Ser Asn Asp

     3315          3330          3345
TCG AGA AAT GTA TTA GTG GCT CCA ACT TCA ATG TTG GAT CAA AGT TTA TCT TCT
Ser Arg Asn Val Leu Val Ala Pro Thr Ser Met Leu Asp Gln Ser Leu Ser Ser

3360          3375          3390       3400       3410
CTT CAA TTT GCT AGA GCA GCT TAA TTTTTAATG  ATTGGCAACT  CTATATTGTT
Leu Gln Phe Ala Arg Ala Ala  *

      3420       3430       3440       3450       3460       3470
TCACACATTA TAGAGTTGCC GTTTTATTTT ATAAAAGGAG ACAATATGGA AGCTAACCAT

      3480       3490       3500       3510       3520       3530
CAAAGGAATG ATCTTGGTTT AGTTGCCCTC ACTATGTTGG CACAATACCA TAATATTTCG

      3540       3550       3560       3570       3580       3590
CTTAATCCGG AAGAAATAAA ACATAAATTT GATCTTGACG GAAAAGGGCT TTCTTTAACT

      3600       3610       3620       3630       3640       3650
GCTTGGCTTT TAGCTGCAAA ATCGTTAGCG TTGAAAGCGA AACACATTAA AAAAGAGATT

      3660       3670       3680       3690       3700       3710
TCCCGCTTAC ACTTGGTGAA TTTACCGGCA TTAGTTTGGC AAGATAACGG TAAACATTTT

      3720       3730       3740       3750       3760       3770
TTATTGGTAA AAGTGGATAC CGATAATAAC CGCTATTTAA CTTACAATTT GGAACAAGAT

      3780       3790       3800       3810       3820       3830
GCTCCACAAA TTCTGTCACA AGACGAATTT GAAGCCTGCT ATCAAGGGCA GTTAATTTTG

      3840       3850       3860       3870       3880       3890
GTCACGTCCA GAGCTTCCGT AGTAGGTTAA TTAGCAAAGT TCGATTTCAC CTGGTTTATT

      3900       3910       3920  3925
CCGGCGGTGA TCAAATACCG AAAAATCTTT CTAGA
                                  X
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FIG. 4. (*Continued*)

insert DNA revealed two ORFs coding for proteins of molecular sizes 19.8 and 101.9 kDa. Two proteins of corresponding molecular sizes can be detected in minicell-labeling experiments, suggesting that the ORFs represent two genes. Further, subcloning of the insert DNA on pPH5 showed that expression from both genes is required for leukotoxic activity. Because of the homologies detected with the *E. coli* alpha-hemolysin *hlyC* and *hlyA* genes, we propose that the leukotoxin genes be designated *lktC* and *lktA*, respectively, to follow existing convention and avoid future confusion.

Because there are considerable similarities between the leukotoxin and the alpha-hemolysin, a number of the established characteristics of the alpha-hemolysin (3) may be applied to the leukotoxin. For example, it has been shown that expression from both *hlyC* and *hlyA* is required for haemolytic activity (24) and that HLYC functions in the activation of HLYA (22). Because it was observed that expression of both leukotoxin genes is required for activity, a similar relationship between LKTC and LKTA may also exist. The leukotoxin is a secreted protein from *P. haemolytica* A1 (33), yet no signal sequence was identified at the amino terminus of LKTA as in the case of HLYA (7, 8). In the alpha-hemolysin locus, two additional functions located downstream from *hlyA* which are required for the secretion of HLYA have been identified both genetically (37) as well as directly on the DNA sequence (8). Furthermore, it has been reported that the sequences at the carboxy terminus of HLYA are involved in the secretion of the protein (9, 18, 23). It can be seen in the present analysis that the carboxy terminus of LKTA shows little homology to that of HLYA. However, the similarities between the leukotoxin and the alpha-hemolysin suggest that there are probably specific functions involved in the secretion of the leukotoxin as in the case of the alpha-hemolysin. It is apparent that the secretion

FIG. 5. Analysis of the DNA sequence upstream from (a) *lktC* and (b) *lktA*. Symbols: ■, sequences similar to the −35 region; —, sequences similar to the −10 region of the *E. coli* promoter consensus sequences; ▭, sequences which, when transcribed into mRNA, produce sequences similar to the *E. coli* ribosome-binding site. The numbers above the DNA sequences indicate the positions of the nucleotides as shown in Fig. 4.

function of the leukotoxin may be similar but not necessarily identical to that of the alpha-hemolysin. Preliminary sequence analysis of the DNA downstream of *lktA* indicates the presence of a *hlyB*-like coding region which may be involved in the secretion of the leukotoxin (unpublished results). Finally, the alpha-hemolysin has been postulated to act by formation of a single pore in the target cell membrane by a mechanism enhanced by Ca^{2+} ions (1, 13); it is possible that the leukotoxin has a similar mode of action, since its activity is also influenced by Ca^{2+} ions (D. G. Gerbig, Jr., J. S. Foster, R. D. Walker, and R. N. Moore, Abstr. Annu. Meet. Am. Soc. Microbiol. 1987, B-27, p. 29).

Sequence analysis of the insert DNA from pPH5 showed that one of the ORFs, ORF2, corresponding to *lktA*, was missing the sequence coding for the carboxy terminus of the LKTA protein. In this configuration, ORF2 terminates 17 amino acids into the vector sequence of pBR322. After isolation of pPH5A and the examination of the proper termination sequences of ORF2, it was observed that only 12 amino acids were missing from the carboxy terminus of LKTA as expressed from pPH5. Apparently, substitution of the 12 amino acids at the carboxy terminus of LKTA by the 17 amino acids from the pBR322 vector did not alter the activity of LKTA significantly. During the initial isolation of pPH5, recombinant plasmids carrying the same insert DNA cloned in the opposite orientation into pBR322 were also recovered (17). These recombinant plasmids expressed a lower leukotoxic activity (17). In the opposite orientation, ORF2 read much further into the vector sequences and produced a protein significantly larger than LKTA with a reduced leukotoxic activity, suggesting that with regard to toxic activity, the carboxy terminus of LKTA is dispensable.

FIG. 6. Predicted mRNA structure of the transcriptional termination signal distal to *lktA*. This structure consists of a 21-bp stem and loop region, followed by a stretch of uridylate residues. The numbers above the mRNA sequence correspond to the DNA sequence shown in Fig. 4. The final two codons and the termination codon of *lktA* are also shown as references to the location of this structure.

FIG. 7. Homology between the leukotoxin and the alpha-hemolysin coding regions. (a) Genetic organization of the two toxins. The arrows indicate the orientation with respect to transcription. (b) A matrix plot showing DNA sequence homology between the two toxins. The diagonal lines represent regions sharing greater than 70% homology over a window of 30 nucleotides.

When pLKT5 was expressed in *E. coli* minicells, both LKTC and LKTA were detected with the predicted molecular weights. On the other hand, only LKTA was detected in the Western blot analysis of the total cellular proteins from the *E. coli* clones. Because the antiserum used in the analysis was raised against the soluble antigens in the culture supernatant of *P. haemolytica* A1, it is possible that LKTC is an intracellular component involved in the activation or modification of LKTA or that LKTC is a secreted component which is nonantigenic. In the case of the alpha-

hemolysin, HLYC has been suggested to be intracellularly located (37). LKTC may be similar in this respect.

The DNA sequences upstream from both *lktC* and *lktA* showed potential transcriptional and translational initiation sequences similar to the consensus sequences of *E. coli*. Because of the lack of reported *P. haemolytica* sequences, *E. coli* sequences were used for comparison. This analysis suggested that if *lkt*C and *lkt*A were expressed as separate transcriptional units, there would be a certain degree of overlapping between the regulatory regions of the two genes. On the other hand, it is also possible that the two genes are transcribed in a polycistronic mRNA containing both *lktC* and *lktA*. It is not possible to distinguish between the two alternatives until the mRNA expressed from this region is examined. Results from subcloning experiments in which the removal of the promoter-proximal half of *lktC* also abolishes *lktA* expression favor the polycistronic mRNA alternative. In addition, no potential transcriptional termination signal was detected following *lkt*C. A similar mode of expression for the *hlyC* and *hlyA* of the alpha-hemolysin has also been suggested (8).

On the basis of the deduced amino acid sequence, LKTC would be highly basic (predicted isoelectric point of 9.83) and LKTA would be slightly acidic (predicted isoelectric point of 6.27). Similar isoelectric points were also predicted for HLYC (10.08) and HLYA (6.06). If these two pairs of proteins do interact with each other as hypothesized, these predicted isoelectric points may influence the interaction positively. An examination of the amino terminus of both LKTC and LKTA shows no patterns similar to the signal sequences predicted for a number of secreted proteins characterized (20, 35, 36). Since the leukotoxin is normally secreted from *P. haemolytica* A1, an alternative secretory mechanism not involving an amino-terminal signal may be used as in the alpha-hemolysin. Interestingly, when the amino acid content of a number of extracellular proteins from a variety of gram-positive and gram-negative bacteria were analyzed, it was observed that they all contain few, if any, cysteine residues (26). The predicted amino acid sequence of LKTA indicates no cysteine residues in this protein. A similar situation was also observed for the *E. coli* alpha-hemolysin HLYA.

In conclusion, we have determined the nucleotide sequence of the leukotoxin-coding region of *P. haemolytica* A1 and identified two genes which are required for the expression of leukotoxic activity. The identification of the leukotoxin genes is a first step toward a better understanding of the function and expression of the leukotoxin and its possible role in pathogenesis. As a result of the homology observed between the leukotoxin and the *E. coli* alpha-hemolysin, a number of characteristics established for the alpha-hemolysin can be used as the basis for further experimentation on the leukotoxin. Finally, because of the potential economic significance of the cloned leukotoxin genes, a patent has been filed by the University of Guelph for the leukotoxin-encoding plasmids and the leukotoxin genes in the development of a subunit vaccine against bovine pneumonic pasteurellosis.

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ADDENDUM IN PROOF

Recently, Koronakis et al. (V. Koronakis, M. Cross, B. Senior, E. Koronakis, and C. Hughes, J. Bacteriol. **169**:1509–1515, 1987) and R. A. Welch (Infect. Immun. **55**:2183–2190, 1987) also detected DNA sequence homology between the *E. coli* alpha-hemolysin determinant and the hemolysin determinants of *Proteus mirabilis*, *P. vulgaris*, and *Morganella morganii*, suggesting that this determinant is also disseminated to a number of other pathogenic bacteria.

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