

Molecular Characterization of a Leukotoxin Gene from a *Pasteurella haemolytica*-Like Organism, Encoding a New Member of the RTX Toxin Family

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A *Pasteurella haemolytica*-like organism, a new species of bacterium isolated from piglets with diarrhea, secretes a leukotoxin into the culture media. Western blot (immunoblot) analysis indicated that this leukotoxin cross-reacted with antileukotoxin antibody derived from cattle immunized with *P. haemolytica*. Five overlapping recombinant bacteriophages carrying the gene for this 105-kDa polypeptide were identified with a DNA probe containing sequences from the *P. haemolytica lktCA* genes from a *P. haemolytica*-like organism strain 5943 genomic library. Sequence analysis of a region of the cloned DNA revealed two open reading frames encoding proteins with predicted masses of 19.4 and 101.6 kDa. These genes, which we designate *plktC* (*P. haemolytica*-like organism leukotoxin C gene) and *plktA* (*A* gene), respectively, are similar in sequence to the RTX (repeat of toxin) toxin family. The structure of the 101.6-kDa protein derived from the DNA sequence shows three transmembrane domains in the N-terminal part of the protein, 13 glycine-rich repeat domains in the second half of the protein, and a hydrophobic C-terminal part. *plktC* and *plktA* are strongly homologous to *P. haemolytica lktC* and *lktA* genes. However, this leukotoxin kills both BL-3 and pig leukocytes and is not hemolytic.

Pasteurella haemolytica-like (PHL) organism is a gram-negative, nonmotile, beta-hemolytic, pleomorphic, oxidase-positive, and urease- and indole-negative organism which was isolated from pigs with enteritis (32). DNA-DNA hybridization studies showed that this organism could be distinguished from other organisms such as *Pasteurella haemolytica*, *Pasteurella multocida*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Actinobacillus pleuropneumoniae*, and *Salmonella cholerae-suis* (32). Results of these studies suggest that the PHL organism may belong to a new group of organisms of the genus *Pasteurella* (27, 32).

In the past several years, a number of gram-negative bacteria which secrete high-molecular-weight (100,000 to 120,000), calcium-dependent cytotoxic proteins that are immunologically and genetically related to the alpha-hemolysin (HlyA) of *E. coli* have been discovered (3, 6, 7-9, 10, 13-19, 21-24, 36). These toxins have been designated the RTX (repeat of toxin) toxin family on the basis of a series of glycine-aspartic acid-rich nonapeptide repeats found in the carboxyl-terminal third of the toxin protein (38). The genetic determinants for the secreted RTX toxins consist of four genes: *A*, the structural gene for toxin protein; *C*, which is required for "activation" of the toxin prior to secretion; and *BD*, which are essential for the process of secretion. The four RTX genes are typically found in a single transcriptional unit, *CABD*, and are expressed from a common promoter located upstream of the *C* gene (19, 26, 39). However, in *A. pleuropneumoniae* hemolysin determinants (*apxII*), the *BD* gene pairs are lost during the evolutionary process (10).

In this paper, we report that PHL organism also secretes a leukotoxin with a molecular weight (105,000) similar to that

of *P. haemolytica* leukotoxin and the complete nucleotide sequence of *C* and *A* genes. This leukotoxin is less species specific than *P. haemolytica* leukotoxin, because it kills both BL-3 cells and pig leukocytes.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. *P. haemolytica* 629 and p1148 (a streptomycin-dependent mutant) were described previously (8, 11). PHL organism strains 6451A, 6646, 900B, 5943B, and 6794A were described before (32). *P. multocida* p1059 was isolated from a cow that died of pneumonia (11). All *Pasteurella* strains were grown in brain heart infusion broth. All *E. coli* strains {JM101, *supE thi* $\Delta(lac-proAB)$ F' [*traD36 proAB⁺ lacI^q lacZ* Δ M15]; TB1, *ara* $\Delta(lac-proAB)$ *rpsL* Φ 80*dlacZ* Δ M15 *hsdR17* (*r⁻ m⁺*); LE392, *hsdR514* (*r⁻ m⁺*) *supE44 supF58 lacY1 galK2 galT22 metB1 trpR55*; P2392, a P2 lysogen of LE392} were cultured in Luria broth (30) containing the appropriate antibiotics when necessary. Vectors lambda-Dash, M13mp18, M13mp19, and pHG165 were as described previously (9, 29). The intact *plktCA* genes from lyfc34 were subcloned into pHG165 (37) as an *EcoRI* fragment to form pYFC93.

SDS-PAGE and Western blotting. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting (immunoblotting) were performed as described previously (8, 9), using culture supernatants (5 ml) concentrated by the chloroform-methanol-water system (40), and resuspended in 150 μ l of sample buffer. After being boiled for 2 min, samples (15 μ l) were subjected to SDS-PAGE. Immunoreactive proteins were visualized with bovine anti-leukotoxin and an anti-bovine immunoglobulin G second antibody conjugated to alkaline phosphatase (8).

Construction of a genomic bank of PHL organism DNA in

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λ -Dash. PHL organism chromosomal DNA from strain 5943B was isolated as described previously (9, 35) and partially digested with *Sau3A*. The digested DNA was fractionated by sedimentation through a 10 to 40% sucrose gradient (8, 9), and fractions containing 9- to 20-kbp fragments, as judged by agarose gel electrophoresis, were pooled and concentrated by ethanol precipitation to a final concentration of 100 μ g/ml. λ -Dash was cleaved with *Bam*HI and treated with alkaline phosphatase to remove terminal phosphatase. After phenol extraction and concentration by ethanol precipitation, the vector DNA was mixed with size-selected PHL organism DNA at a molar ratio of 1:4 and treated with T4 DNA ligase for 16 h at 15°C. The ligated DNA mixture was packaged into λ particles with a commercially available in vitro packaging kit (Gigapack plus; Stratagene, La Jolla, Calif.). The phage titers were determined and amplified on P2392.

Phage library screening for PHL organism leukotoxin gene. The bacteriophage library was screened by hybridization with a probe containing the *lktCA* genes from *P. haemolytica*. A DNA fragment from pYFC19 (8) containing the *lktCA* genes was labeled with [³²P]dATP by nick translation. Filters were hybridized in 45% formamide–5 \times SSC (20 \times SSC contains 175.3 g of NaCl and 88.2 g of sodium citrate per liter, pH 7.0)–5 \times Denhardt's solution–100 μ g of sheared calf thymus DNA per ml for 12 h at 37°C. Filters were then washed twice with 2 \times SSC–0.1% SDS and twice with 0.2% SSC–0.1% SDS at room temperature. The final wash was with 0.16% SSC–0.1% SDS at 37°C. Plaques which gave positive signals were picked, rescreened, and amplified on P2392.

DNA sequencing and analysis. DNA sequencing was performed by the dideoxy chain termination method (34). Regions from the PHL organism inserted in bacteriophage clone λ yfc34 were subcloned into M13mp18 or M13mp19, and single-stranded phage DNA was prepared by standard procedures (29). The sequencing reactions utilized ³⁵S-dATP, T7 DNA polymerase, and the commercially available Sequenase kit (United States Biochemicals, Cleveland, Ohio). Certain regions of the DNA insert were sequenced directly from the recombinant bacteriophage. In these cases, 1 to 2 μ g of λ yfc34 DNA was mixed with 100 ng of an oligonucleotide primer (prepared by the Analytical and Synthetic Facility, Cornell University) in a total volume of 12 μ l; the mixture was boiled for 5 min and then cooled rapidly on ice. The sequencing reactions were performed with reagents supplied with the Sequenase kit, using the manufacturer's instructions. DNA sequence analysis was performed on a VAX computer with the Genetics Computer Group program package (University of Wisconsin, Madison). The amino acid sequence alignment was carried out with the GAP and LINEUP programs (Genetics Computer Group, University of Wisconsin, Madison), and similarity was calculated by the method of Pearson and Lipman (33).

Southern blotting analysis. PHL organism genomic DNA from different isolates was digested with *Pst*I, electrophoresed through a 0.7% agarose gel, transferred to a nitrocellulose membrane, and probed with an *Xba*I–*Sal*I fragment containing 3,120 bp of partial *pllktCA* genes isolated from λ yfc34. Filter treatment and hybridization procedures were as described above.

Assay of cytotoxic activity. The pig lymphocytes were prepared as described previously (4). The cytotoxic activity of PHL organism leukotoxin against freshly prepared pig lymphocytes and BL-3 cells was quantitated by a tetrazolium (MTT) dye (3-[4,5-dimethylthiazoyl-2-yl]-2,5-di-phe-

nyltetrazolium bromide) reduction assay as described previously (32). BL-3 cells or pig lymphocytes (100 μ l of 2 \times 10⁶/ml) were seeded into 96-well tissue culture plates, and 100 μ l of culture supernatant was dispensed to wells containing cells. All samples were run in triplicate. Controls included cell viability (wells containing cells and Leibovitz L-15 medium) and nonspecific dye reduction (wells containing Leibovitz L-15 medium and culture supernatant but no cells). Plates were incubated for 1 h in a humid atmosphere at 37°C and 5% CO₂. Plates were centrifuged for 10 min at 500 \times g at room temperature, and the supernatant was gently removed. Each well then received 80 μ l of L-15 and 20 μ l of MTT (5 mg/ml in 0.01 M phosphate-buffered saline, pH 7.2), and plates were incubated for 4 h at 37°C and 5% CO₂. Plates were again centrifuged, dye and supernatant were removed, and 200 μ l of acid isopropanol (0.04 N HCl in isopropanol) was added per well to dissolve formazan. The absorbance was read on an enzyme-linked immunosorbent assay plate reader (Bio-Tek EL312) at a reference wavelength of 650 nm and a test wavelength of 570 nm, with the threshold set at 1.99. Both BL-3 cells and fresh pig leukocytes undergo similar characteristic morphological changes upon exposure to the active leukotoxin from PHL organism; for BL-3 cells, they are also similar to changes induced by *P. haemolytica* leukotoxin (8).

Nucleotide sequence accession number. The DNA sequence of *pllktC* and *pllktA* has been submitted to GenBank and assigned accession number L12148.

RESULTS

Western blotting analysis. To determine whether the PHL organism also secreted the leukotoxin, the culture supernatants from two *P. haemolytica* strains, *P. multocida*, and five strains of PHL organism were analyzed by Western blot, using bovine antiserum raised against *P. haemolytica* leukotoxin. A cross-reacting polypeptide species of 105 kDa was identified (Fig. 1A). Thus, the PHL organism leukotoxin, which we designated PILkt, is immunologically related to *P. haemolytica* RTX toxin.

Cloning the *pllkt* locus. We screened a PHL organism genomic library constructed in the phage vector λ -Dash with a DNA probe derived from pYFC19, a plasmid carrying the *lktCA* locus (8). Five clones were isolated which overlapped each other (Fig. 2). Except λ yfc36, all other clones expressed a 105-kDa polypeptide detected by Western blot with the antileukotoxin antibody (Fig. 1B). λ yfc36 produced a truncated polypeptide of 70 kDa (Fig. 1B, lane 4). The fact that this clone expressed a truncated toxin provided a location and orientation for the putative *pllkt* locus within the cloned DNA. Southern blot analysis with an *Xba*I–*Pst*I fragment, which maps to the toxin determinants as judged by DNA sequencing, showed that no detectable rearrangement occurred during the cloning procedure (Fig. 3). Despite the fact that all four clones which produced the full-length leukotoxin were identified, no cytotoxic activity could be detected in any of the phage lysates (data not shown).

DNA sequence of *pllktCA* genes. A 4-kbp region indicated by the truncated clone was subjected to DNA sequence analysis (Fig. 4). As in the case of the RTX loci, there is a small open reading frame preceding the toxin open reading frame, presumably encoding the *pllktC* gene (Fig. 4). The *pllktCA* genes are more closely related to *lktCA* from *P. haemolytica* than to other members of the RTX toxin family. Table 1 summarizes the similarities between *pllktCA* and the

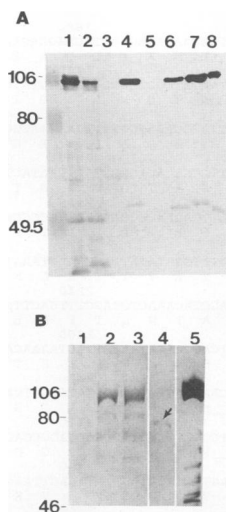


FIG. 1. Immunoblot of culture supernatants incubated with anti-*P. haemolytica* leukotoxin antibodies and expression of the *pllktA* gene. (A) Western blot analysis of culture supernatants from *P. haemolytica*, *P. multocida*, and PHL organism, using bovine anti-leukotoxin serum as the first antibody: *P. haemolytica* 629 (lane 1), *P. haemolytica* p1148 (lane 2), *P. multocida* p1059 (lane 3), PHL organism strains 6451A, 6646, 900B, 5943B, and 6794A (lanes 4 to 8, respectively). (B) Western blot analysis of antigenic proteins expressed from recombinant bacteriophage. Lysates were from *E. coli* LE392 infected with λ -Dash (lane 1), λ ycf34 (lane 2), and λ ycf35 (lane 3). The truncated protein (λ ycf36, lane 4) is indicated by an arrow. Leukotoxin secreted by PHL organism is shown in lane 5. Prestained molecular markers (Sigma Chemical Co., St. Louis, Mo.) and their apparent molecular weights (10^3) are shown on the left.

other RTX *CA* genes for which sequence information is available.

The *pllktCA* sequence was examined for *E. coli* promoter-like sequences by the homology score method (30). There were two sequences, TAATCT and TAAAAT, similar to the TATAAT consensus promoter sequence (-10 region) and one sequence, TTGATT, similar to the consensus RNA polymerase-binding site (-35 region) (30) proximal to *pllktC* (Fig. 4). Upstream of the start codon of *pllktC*, there is a potential ribosome-binding site (Fig. 4). A ribosome-binding site lies proximal to *pllktA* (Fig. 4). A sequence very similar to the rho-independent transcriptional terminator of *E. coli* downstream from *pllktA* was also observed (Fig. 4).

We analyzed the hydrophobicity of the deduced amino acids of PILktA and its potential membrane-spanning regions by the method of Klein et al. (20). The analysis identified three potential transmembrane regions on PILktA between amino acids 154 to 170, 312 to 333, and 393 to 414 (Fig. 4).

Southern blotting analysis. To demonstrate the distribution of these genes (*pllktCA*) among different field isolates of PHL organism, the 3,120-bp *Xba*I-*Sal*I fragment from λ ycf34 was purified, nick translated with [32 P]dATP, and used as a hybridization probe on genomic DNA of PHL organism field isolates in Southern blots. Except PHL organism strain 6646, all other strains contain two unique signals, 2.5 and 9.2 kbp (Fig. 3).

Expression of cytotoxic activity in *E. coli*. The *pllktCA* region from λ ycf34 (Fig. 2) was subcloned into vector pHG165 (36) as an *Eco*RI fragment yielding a plasmid, pYFC93 (*pllktCA*). This clone is likely to be expressed from

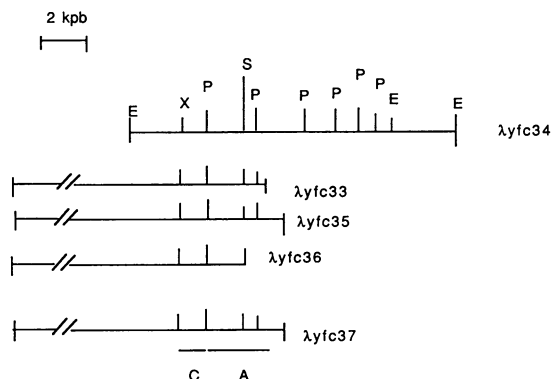


FIG. 2. Restriction maps of the PHL organism leukotoxin clones. *Eco*RI sites derived from the vector flank the inserts of each clone. Except clone λ ycf36, all clones expressed a 105-kDa polypeptide detected by Western blotting. The locations of the two open reading frames designated *pllktC* and *pllktA* found by sequence analysis are indicated. E, *Eco*RI; P, *Pst*I; S, *Sal*I; X, *Xba*I.

the PHL organism promoter and to contain *pllktBD* genes because we detected toxin in the media (Fig. 5). Using the culture supernatant of PHL organism strain 5943B, we detected 42.7 and 40.6% cytotoxicity per 100 μ l of culture supernatant with BL-3 cells and pig lymphocytes, respectively (Table 2). Using the culture supernatant of *E. coli* harboring pYFC93, we detected 23.3 and 21.4% cytotoxicity per 100 μ l by using either BL-3 cells or pig lymphocytes, respectively (Table 2). The cytotoxicities of *P. haemolytica* 629 and other strains of PHL organism leukotoxins are shown in Table 2. However, with culture supernatant from *E. coli* harboring vector pHG165, we did not detect any toxin activity (Table 2). The leukotoxic activity from *E. coli* harboring pYFC93 could be neutralized by cattle anti-*P. haemolytica* leukotoxin antibody, as is the case with the leukotoxin from PHL organism (data not shown).

DISCUSSION

We have identified a new species of bacterium from pigs with diarrhea which secretes a toxin that can kill BL cells as well as fresh pig lymphocytes. This toxin could be detected by Western blot analysis by using neutralizing antisera to the *P. haemolytica* leukotoxin. This suggests that PHL organism produces a leukotoxin which is a new member of the RTX toxin family. A PHL organism genomic library was constructed in the replacement vector λ -Dash and screened with a DNA probe derived from pYFC19 which contains *lktCA* genes of *P. haemolytica* (8). A series of five overlapping clones which produced a 105-kDa polypeptide when expressed in *E. coli* LE392 were identified. One clone, λ ycf36,

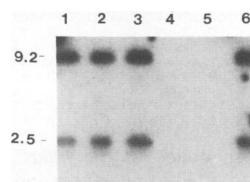


FIG. 3. Southern blotting analysis of five strains of PHL organism (lane 1, 6451A; lane 2, 6794A; lane 3, 900B; lane 4, *P. multocida* p1059; lane 5, 6646; lane 6, 5943B). The filter was hybridized and washed as described in Materials and Methods.

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380                               -360                               -340                               -
GGCAGCATAATCCTGGAGCCTTAAACTTAAATTAAGTGGCTGATTCAGCAAAATTT
320                               -300                               -280                               -
GCACAAATCAGACCCGTGATTTTATTAGTACCTCATCTCTTTATTGTAATAATTTATCC
260                               -240                               -220                               -
TTACAAAACGATACATCTCTTAAACTTTCTTAAATAAAATGAAAAGCAAATATTACAT
200                               -180                               -160                               -
TAATTTTACAATGTAATTAATTTTGTTTTGGCCATTTGCTTAAAGTATTT
140                               -120                               -100                               -
TATTTTGCATTAATTTACATGAAAGCCAAAACACAAATTTAAACAATTTAAACAATA
-80                               -60                               -40                               -
AAAATCCTGTGGTAAAGTACAGTGTATTAATATATATGCTTAAATTTGGATCTAATCTA
          ^
          ^
          ^
-20                               1                               20
GAATCATTATCGAGTGTGAATTAATGAATCAACATTACTTTAATCTATTGGGAAATATTAC
          M N Q H Y F N L L G N I T
40                               60                               80                               1
TTGGTATTGGATGAATCACCCTTTCATAGAGAGTGGAGCTGTGAGCTATTGGCAGCAG
W L W M N S P L H R E W S C E L L A R N
00                               120                               140                               1
TGTGATTCGGCAATGAAATCAACAATATATGCTACTTATTGATAATGATGTTCCAA
V I P A I E N Q Q Y H L L I D N D V P I
60                               180                               200                               2
CGCATATTGCAAGTTGGCAGATTTAGCCTTGAAGTGAAGTAAAGTATTAAGGATTA
A Y C S W A D L S L E T E V K Y I K D I
20                               240                               260                               2
TAGTTCATTAACCCGGAAGATGGCAGTCTGGCAGTAGACGTTGGATTTATTGGGTT
S S L T P E E W Q S G D R R W I I D W V
80                               300                               320                               3
AGCACCATTGGGCACTTCTCAACTTATAAAAAATGTGTCAAGAAATACCTGATTACT
A P F G H S Q T L L I K N V S E I P D Y A
40                               360                               380                               4
TCGTCAGATCTATACGCTTTTATCCAAAACAAAAGAACTGGCAAAATGGCATTMTAA
R Q I Y T L L S K T K R T G K I A Y F K
00                               420                               440                               4
AGGGGGAACTTAGTAAAAAAGCAGAAAACGTTTCGATACATATCAAGAAAGGCT
G G N L D K K K T A K K R F D T Y Q E R L
60                               480                               500                               5
GGGGCAGCTCTTAAAAATGCTTTAATTTTCTAAATAAAAGGACATCCTTATGGGT
G A A L K N E F N F T K M G
20                               540                               560                               5
AAACTGTGTAATTTCAACAAACTTAAAAATTCATGCAATCCGCTTTGCATAAAACA
K L A N I S T N L K N S L Q S G L H K T
80                               600                               620                               6
GGGCAATCTTTAAACCAAGCCGCTCAATTTTAAAGCCGGAGCGAAAAGCTCATTCTC
G Q S L N Q A G Q S L K A G A K K L I L
40                               660                               680                               6
TATATTCCAAAAGATTATGAATATGATTCAGGAAGAGAAACGGTTTACAGGATTTAGCT
Y I P K D Y E Y D S G R G N G L Q D L V
00                               720                               740                               7
AAAGTGCAGAAATTAGTATGGAAGTACAAGAGAAAGAGCGCTAATGGTATTGCTACC
K A A E D L G I E V Q R E E R N G I A T
60                               780                               800                               8
GCTCAAAACAGTTAAGTACAATCAAAATATCTTGGGTTAGCGAGCTGGAGTTGTA
A Q N S L S T I Q N I L G F S E R G V
20                               840                               860                               8
TTGTCTGCTCAACTTGATAAACTGCTTCAAAAATACAAAATCAGTAAGCAGCAGGT
L S A P L D K L L Q K Y K I S K A P G
80                               900                               920                               9
TCATCAGAAAATGATGCTTAAAATTTGGGTAGTCACAACTTTATTATTCGGTATTCAA
S E N V A K N L G N A Q T L L S G I Q
40                               960                               980                               10
TCTATTTAGGCTCAGTATGGCCGCTATGGATTAGATGAACTTGAATAAAAGGA
S I L G S V M A G M D L D E I L K N K G
00                               1020                               1040                               10
AGTGAATCTGATTAGCAAAGCTGGTTAGAAATTAAGTAACTAATTCGTTAATGAAATATT
S E L D L A K A G L E L T N S L I E N I
60                               1080                               1100                               11
GCAAAATCTGTTCAACGCTGATCTTTTTCAGAACAAATAGCCAATTAGTACTAG
A N S V Q T I D T F S E Q I S Q L G T K
20                               1140                               1160                               11
TTACAAAATGTAAGTTAGTACTTTAGGAGATAAACTTAAAACTTTAGTGGCTTC
L Q N V K G L G T L G D K L K N F S G F
80                               1200                               1220                               12
AGTAAAGCTGCTTGCCTTAGAAGTAAATCCCGTTTCTTCTGTCGAACAGCAGCT
S K A G L G L E V I S G L L S G A T A A
40                               1260                               1280                               13
CTTGTCTTCAGATAAAATCCCTCTACAGATAGGAAGTAGTGTGGCTTTGAGCT
L V L A D K N A S T D R K V G A G F E L
00                               1320                               1340                               13
GCACCAAGTGTGTGTAACATCACAAAGCCGTTCTCTTATATTATAGCACAGGCT
A N Q V V G N I T K A V S S Y I L A Q R
60                               1380                               1400                               14
GTTCCGCGGGTTTATCTAATCAGGCCAGTGTGAGCATTAAATGCTTCTACTGTGCA
V A A G L S N T G P V S A L I A S T V A
20                               1440                               1460                               14
CTTGCTATTAGTCCGCTTTCAGGAATTGCAGATAAATTTAACAATGCTAAAAGCA
L A I S P L A F A G I A D K F N N A K A
80                               1500                               1520                               15
CTTGAAAGTTATGACAGAGATTTAAAAAATAGGCTATGAGGGGATGTTTACTCGCT
L E S Y A E R F K K L G Y E G D S L L A
40                               1560                               1580                               16
GAATPACCAAGGAAACAGTACGATAGTCTTCTGTAACCGCGGTTAATACTGCATTA
E Y Q R G T G T I D A S V T A V I D A L
00                               1620                               1640                               16
GCTGCAATTCAGTGGCGTTTCAGCCGAGCAGCCGTTCTCTAGTCGGCGCACCGATT
A A I S G G V S A A A A G S L V G A P I
60                               1680                               1700                               17
GCTCTACTTGTTCGTATCCCGGAATTTCTCAACTTCTCAAACTACTTAAACAA
A L L V S G I T G F I S T I L Q Y S K Q
20                               1740                               1760                               17
GGGATGTTGAGCATGTAGCGAATAAAATCAGGATAAATTTGGATTTGGAGAAAAAA
A H F E H V A N K I H D K I V D W E K K
80                               1800                               1820                               18
CATAAAGGCAAACTACTTCGAAAATGGTATGACTCTGCTTATTAGCCGATCTTCAA
H N G K N Y F E N G Y D S R Y L A D L Q
40                               1860                               1880                               19
GACAAATGCGTCAGTTCAGAAATCTCAATAAAGAACTACAAGCAGAACGGTTATCCGG
D N M R Q L Q N L N K E L Q A E R V I R
00                               1920                               1940                               19
ATTACGCAACGCAATGGGATAATAATTTGGTAACTCGCTGGTATCAGCCGATTAGGT
I T Q Q Q W D N N I G N L A G I S R L G
60                               1980                               2000                               20
GAAAAGTAAATGAGCGAAAAGCTTATGCAGATGCTTTGAAGAAGGCAAACTCATAAAA
E K V M S G K A Y A D A F E E G K L I K
20                               2040                               2060                               20
GCAGATACATTTGTACAATTAGATTCTGCCACGGGATCAATACTAGCAAGTGTGAT
A D T F V Q L D S A T G V I N T S K S D
80                               2100                               2120                               21
AATGTTAAACTCAGCATATTTTATTGAAACGCCACTACTTACCCAGGGGTAGAAAAT
N V K T Q H I L F R T P L L T P G V E N
40                               2160                               2180                               22
CGTGAGCGTATTCAAACCTGGTAAATATGAGTATATTACCAAATTAATTAACCGGTGA
R E R I Q T G K Y E Y I T K L N I N R V
00                               2220                               2240                               22
CACAGCTGAAAATTAAGTATGAGCTCAAACTACTCTTACCTTTGACTTGAATAATGGTGT
D S W K I T D G A T N S T F D L T N V V
60                               2280                               2300                               23
CAACGATTGGTATTGAATTAGATCAGCAGATAATGTTACTAAAACAAAAGAGACTAA
Q R I G I E L D H A D N V T K T K E T K
20                               2340                               2360                               23
ATTATTGCAAACTAGTGTAGGCAATGATGATTATTGGTTGAGGCAAACTGAA
I I A N L G D G N D D V F I G S G T T E
80                               2400                               2420                               24
GTTGATGGTGAACCGCTTTGATCGCGGCTATTATGCGGAGGCGACTACGGTGCATTA
V D G G N G L D R V H Y S R G D Y G A L
40                               2460                               2480                               25
ACTATTGATCAACGAATGAATCAGTCAAGCTAGTATACAGTAAKCGTTCCTGTGAA
T I D A T N E S V Q G S Y T V K R F V E
00                               2520                               2540                               25
ACTGGTAAAGCAATGCATGAAGTAACTCAACTCAATCTGTTTATGTTAGCGCGGAA
T G K A L H E V T A T Q S V L V G S R E
60                               2580                               2600                               26
GAAAATAATGAGTATCGTCACAGTAATAACACAGCATGCTGGTTACTATACAGAT
E K I E Y R H S N N T Q H A G Y V T T D
20                               2640                               2660                               26
ACTTTAAAGTCTGTGAGGAAATTTGGTACTTACGCAATGATCTTTAAAGGTAGT
T L K S V E E I I G T S R N D I F K G S
80                               2700                               2720                               27
AATTTGATGATGCTTCCATGCGCGCTGATGCTGTGATTAACATTGACGTAATGCAGGC
K F D D A F H G G D G V D N I D G N A G
40                               2760                               2780                               28
AATGACCGCTTATTTGGCGGAAAGCTTATGATATTATGATGGCGGTTAGTGTGATC
N D R L F G G K G F D I I D G G D G D D
00                               2820                               2840                               28
TTTATCGATGGCGCTCAAGGAGATGATATCTTACACGGCGCAAGGCAATGATATCTTG
F I D G G Q G D D I L H G G K G N D I L
60                               2880                               2900                               29
TGCACCGTCAAGGTTGGCAATGATTCAATAGCGACTTGGCGGCAATGATGATTACT
C T V K G G N D S I S D S G N D R L S
2940                               2960                               29
TTCCGGGACTCAAATCTTAAAGATTGGACCTTGAAGAAAGTAAACCACCCTTATGATC
F A D S N L K D L T F E K V N H H L M I
80                               3000                               3020                               30
ACTAATGCTGAAAAAAGAAAGTAACTATTCAAACCTGGTCCGCTGAAGCCGATATGCT
T N V K K E K V T I Q N W F R E A A D Y A
60                               3060                               3080                               31
AAAATCTGTCATAATTAACAGCAACCCGACGAAATAATGAAGAAATCATTGGTCA
K T V H N Y Q A T A D E K I E E I I G R
00                               3120                               3140                               31
CAAGGTGAGCGTATTACCCTAAGCAATTTAGTGTAGCTGAAAGGTTAAAGGTAA
Q G E R I T S K Q I D E L I E K G K G K
60                               3180                               3200                               32
ATTGATCAGAGTGAATTTGGAGAAATTTGCTGAAAGCAATGCTTACTCAAAGAAAGTAA
I D Q S E L E R I A E S S A L K E S K
20                               3240                               3260                               32
TTTGCTTCAATAGCTTAAATAACTTGTTCATCTGAGCGGATTTGCTCTTCAAC
F A S N S L N K L V S S G A F A S S N
80                               3300                               3320                               33
GATAACAGAGTGGGCTTGGCGTTCCATACATCATTTGATGAACATCCCAATCTGTACA
D N R V G L G V P T S L A E H T Q S V Q
40                               3360                               3380                               34
TTTGTAAGCAGCTTAAATTTTAAATGTTTAGCAACTCTATATGTTTACGCCATTAT
F V R A A ----->
00                               3420                               3440                               34
GGAGTTGCTATTTTATTTTTAAAGGATTTATGGAAGTTAATCATCAAGCCAATT
-----*****
M E V N H Q S Q L
60
GATCTTGGAT
I L D

```

FIG. 4. Nucleotide sequence of the *plktCA* region and the predicted amino acid sequences of the PILkTc and PILkTA proteins. Promoterlike regions proximal to *plktC* are indicated by the symbol ^ directly beneath the nucleotide sequence. Potential ribosome-binding sequences preceding *plktC* and *plktA* and immediately after *plktA* are indicated by an underline. A potential rho-independent transcription terminator and poly(T) track-distal *plktA* are indicated by <-> and ***, respectively. The three transmembrane segments are doubly underlined within the *plktA* amino acid sequence. The glycine-rich repeated sequences are underlined within the PILkTA sequence.

TABLE 1. Characteristics of the PILkTC and PILkTA proteins and their gene

Characteristic ^a	<i>pillkA</i> gene or protein	
	C	A
Homology (%) to the leukotoxin locus of <i>P. haemolytica</i> (<i>lkt</i>)		
Nucleotide sequence	86.7	75.7
Amino acid sequence	86.0	86.8
Homology (%) to the hemolysin locus of <i>A. pleuropneumoniae</i> (<i>apxI</i>)		
Nucleotide sequence ^b	68.6	58.8
Amino acid sequence ^c	75.5	65.4
Homology (%) to the hemolysin locus of <i>A. pleuropneumoniae</i> (<i>apxII</i>)		
Nucleotide sequence	63.3	66.8
Amino acid sequence	69.7	76.7
Homology (%) to the hemolysin locus of <i>E. coli</i> (<i>hly</i>)		
Nucleotide sequence	58.1	60.3
Amino acid sequence	66.0	66.2
Homology to the hemolysin locus of <i>A. actinomycetemcomitans</i> (<i>aalkt</i>)		
Nucleotide sequence	62.7	58.4
Amino acid sequence	65.2	64.2
Length in amino acid residues	165	947
M_r	19,400	101,600
pI	8.69	5.49

^a The sequences were taken from the following sources: *apxIA* (15), *apxIIA* (9), *hly* (14), *lkt* (18, 24; our unpublished data), *aalkt* (22, 23).

^b Percent identical residues.

^c Percent identical residues assuming that the following amino acid pairs are equivalent: I and V, S and T, E and D, K and R, and F and Y.

produced a 78-kDa polypeptide which is a truncated form of the 105-kDa protein (Fig. 1B, lane 4).

DNA sequence analysis of a 4-kbp region from clone *lyfc34* indicated the presence of two open reading frames which we designated *pillkC* and *pillkA*. These encode polypeptides of 165 and 947 amino acids, respectively. In addition, there is a potential third open reading frame in the cloned DNA beginning at position 3431 of the sequenced region (Fig. 4). We suspect that this represents the amino-terminal coding region of a putative *pillkB* gene and that a fourth gene, *pillkD*, will lie distal to *pillkB*.

We could not detect leukotoxin activity in lysates prepared by infecting *E. coli* with any of the bacteriophage clones. This is similar to the *A. pleuropneumoniae* hemolysin gene clones, as reported previously (9). When the *pillkCA* genes were subcloned into a multicopy plasmid vector, the recombinant plasmid expressed the 105-kDa protein (Fig. 6) with leukotoxin activity (Table 2). Since the leukotoxin was secreted into media, we suspect that this clone contains the complete gene cluster (*pillkCABD*).

The predicted PILkTC and PILkTA proteins have 86.6 and 86.8% similarity with the corresponding LktC and LktA proteins from *P. haemolytica* leukotoxin determinant. The PILkTA leukotoxin, as is the case with the other RTX toxins, does not have a classic signal sequences at its amino

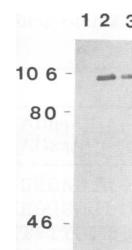


FIG. 5. Expression of the *pillkA* gene from a multicopy plasmid. Western blot analysis of the culture supernatant of TB1 carrying vector pHG165 (lane 1), pYFC93 (lane 3), and PHL organism strain 5943B (lane 2) is shown. The first antibody was bovine antileukotoxin antibody. The apparent molecular weights (10^3) of prestained standards are shown on the left.

terminus. Instead, the predicted amino terminus is rich in serine, threonine, and lysine (13 of the first 39 residues) and has the capability of forming a positively charged, amphiphilic α -helix, as do the amino termini of other RTX toxins (18). There are three transmembrane domains in the N-terminal part of the protein (Fig. 4). These structural features have been reported to facilitate the interaction of this class of lytic toxins with target membranes (18). There are 13 glycine-rich repeats in the second half of the PILkTA (Table 3). The glycine-rich repeats have been reported to be responsible for cell binding (2, 12, 25, 28). However, PILkTA (this study), LkTA (8, 18, 24), and AaLkTA (22, 23) and ApxIIIA (6) are potent leukotoxins with no hemolytic activity; they contain 13 glycine-rich repeats for the former two, 17 for AaLkTA, and 16 for ApxIIIA (Table 3). Despite the strong homology between PHL organism and *P. haemolytica* leukotoxin, the former is less specific than the latter since the former kills both BL-3 and pig leukocytes. A further characterization of these two toxin genes is under way in our laboratory.

In summary, the DNA sequencing results have shown that PILkTA is a member of the RTX toxin family. The level of similarity between LkTA and PILkTA suggests that they

TABLE 2. Cytotoxic activity of culture supernatants from *P. haemolytica*, PHL organism, *E. coli* harboring pYFC93, and *E. coli* harboring pHG165 (vector)

Culture supernatant	% Cytotoxicity	
	BL-3 cells	Pig lymphocytes
<i>P. haemolytica</i> 629 ^{a,b}	85.3	0 ^c
PHL organism ^{a,b}		
5943B	42.7	40.6
6646	0 ^c	0 ^c
6451A	41.2	40.7
900B	47.3	46.4
6794A	40.3	40.5
pYFC93 ^{b,d}	23.3	21.4
pHG165 ^{b,d}	0 ^c	0 ^c

^a Assay was performed with 100 μ l of late-log-phase supernatant from culture of *P. haemolytica* 629 and PHL organism strains 5943B, 6646, 6451A, 900B, and 6794A grown in brain heart infusion broth.

^b Percent cytotoxicity = [1 - (optical density of culture supernatant-treated cells/optical density of untreated cells)] \times 100.

^c These samples were identical to controls containing cells and media only.

^d Assay was performed with early-stationary-phase supernatant from the *E. coli* host, TB1, harboring the indicated plasmid grown in Luria broth with ampicillin (50 μ g/ml).

TABLE 3. Amino acid residues within the glycine-rich repeated domains of PILkTA ApxIA (Hly1A), ApxIIA (AppA), ApxIIIA, LktA, aaLktA, and HlyA

Domain	Sequence ^a						
	PILkTA	AppIIA (ApxIIA)	LktA	AaLktA	ApxIIIA	HlyIA (ApxIA)	HlyA
1	NLGDGNDV	NLGAANDNV	KLGEEDNV	FVGSSTIV	HLGNNDKV	YAGNGHVA	HLGDGDDV
2	VDGGGLDR	IDGGGDHNR	IDGGEGYDR	NAGDGYDVV	HAGEGHVV	DTGYLTFDG	YAGKGDVV
3	HAGYYTDT	HTGYVTDS	HAGYYTKDT	NGKFAHDE	DTGLLVIDG	GNGIRAKDE	DTGYLTIDG
4	IIGTSRNDI	LIGTTRADK	IIGTSHNDI	IIGSTLRDK	EIGSNQRDE	IIGSNRDKK	IIGSQFNDI
5	FKGSKFDDA	FFGSKFADI	FKGSLFMDA	FYGSKFNDV	FKGSKFRDI	FFGSRFTDI	FKGSKFDDV
6	FHGGDGVND	FHGADGDDH	FNGDGVDA	FHGHGDDL	FHGADGDDL	FHGAKGDDE	FHGGNGVDT
7	IDGNAGNDR	IEGNDGNDR	IYNDGNDR	IYGYDDDR	LNGNDGDDI	LYGNDGNDI	IDGNDGDDH
8	LFGGKGFDI	LYGDKGNDT	LFGGKGGDI	LYGNDGNDI	LYGDKGNDI	LYGGDGDNDI	LFGGAGDDV
9	IDGGDGDV	LSGGNGDDQ	LDGGNGDDI	IHGGQGNK	LRGNDGNDQ	IHGGDGNNDI	IDGGNGNNDI
10	IDGGQGGDI	LYGGDGNDK	IDGGKGNDL	LYGGAGNDR	LYGGEGDDK	LYGGNGNDR	LVGGTGNDI
11	LHGGKGNDI	LIGGAGGNY	LHGGKGGDI	LFGEYGNNY	LLGGNGNNY	LIGGKGNNDI	ISGGKNDI
12	VGGGNDIS	KTGDGNDIS	RKGDGNDII	LDGGEEDDI	LSGGDGNDE	LNGGDGDDE	LNGGDGDDE
13	DSGGNDRLS	DSGGQDKLA	TDSGNDKDL	LEGGNGSDI	LRGGKGGDK	LLGGAGNDI	LSGGKGNNDI
14				LYGSSGNDK	LYGSSGNDL	LYGSDGTNL	LYGSSGNDL
15				LFNGQGGDL	LDGGEENDY	FDGGVGNNDI	LDGGEENDL
16				LDGGEEDDQ	LEGGDGSDF	IYGLGKDI	LKGGYGNNDI
17				LAGGEGNDI			

^a Residues were taken from the following sources: PILkTA (this study), ApxIIA (9), LktA (18), AaLktA (22), ApxIIIA (6), ApxIA (15), and HlyA (14).

diverged only recently. In *P. haemolytica*, almost all of the strains contain this leukotoxin determinant (5). In contrast, not all strains of PHL organism carry the leukotoxin determinants (this study). Whether the PHL organism leukotoxin gene cluster is derived from *P. haemolytica* is unknown. The *A. pleuropneumoniae* serotype 7 hemolysin determinants are flanked by identical direct repeats (1). The presence of these repeats allows participation of this DNA region in homologous recombination. Examination of the flanking regions of the PHL organism toxin determinants for the presence of identical repeats will enable us to determine whether the PHL organism toxin determinants could be mobile by using a similar mechanism.

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