# Nucleotide Sequence of the Hemolysin I Gene from Actinobacillus pleuropneumoniae

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The DNA sequence of the gene encoding the structural protein of hemolysin I (HlyI) of Actinobacillus pleuropneumoniae serotype 1 strain 4074 was analyzed. The nucleotide sequence shows a 3,072-bp reading frame encoding a protein of 1,023 amino acids with a calculated molecular size of 110.1 kDa. This corresponds to the HlyI protein, which has an apparent molecular size on sodium dodecyl sulfate gels of 105 kDa. The structure of the protein derived from the DNA sequence shows three hydrophobic regions in the N-terminal part of the protein, 13 glycine-rich domains in the second half of the protein, and a hydrophilic C-terminal area, all of which are typical of the cytotoxins of the RTX (repeats in the structural toxin) toxin family. The derived amino acid sequence of HlyI shows 42% homology with the hemolysin of A. pleuropneumoniae serotype 5, 41% homology with the leukotoxin of Pasteurella haemolytica, and 56% homology with the Escherichia coli alpha-hemolysin. The 13 glycine-rich repeats and three hydrophobic areas of the HlyI sequence show more similarity to the E. coli alpha-hemolysin than to either the A. pleuropneumoniae serotype 5 hemolysin or the leukotoxin (while the last two are more similar to each other). Two types of RTX hemolysins therefore seem to be present in A. pleuropneumoniae, one (HlyI) resembling the alpha-hemolysin and a second more closely related to the leukotoxin. Ca<sup>2+</sup>-binding experiments using HlyI and recombinant A. pleuropneumoniae prohemolysin (HlyIA) that was produced in E. coli shows that HlyI binds  ${}^{45}Ca^{2+}$ , probably because of the 13 glycine-rich repeated domains. Activation of the prohemolysin is not required for Ca<sup>2+</sup> binding.

Actinobacillus pleuropneumoniae, the causative agent of swine pleuropneumonia (36), secretes a hemolytic-cytotoxic activity which is thought to play an important role in virulence of the organism (8, 19, 32). The hemolysin of A. pleuropneumoniae serotype 1 (hemolysin I [HlyI]) is a monomeric protein with an apparent molecular size of 105 kDa which is secreted into the surrounding medium (9). Analysis of the 12 different serotypes of A. pleuropneumoniae has shown that most of them produce a hemolysin of approximately 105 kDa which strongly cross-reacts immunologically with HlyI (10). Serological analysis has revealed that a high proportion of swine possess antibodies against the 105-kDa hemolysin protein (5, 25). Differences in  $Ca^{2+}$ requirements for the hemolytic activity of the various serotypes has indicated, however, that at least two types of hemolysins are produced by the different serotypes. A strongly active HlyI which needs low levels of Ca<sup>2+</sup> for its activity but which requires Ca<sup>2+</sup> in the growth medium to induce its biosynthesis was isolated from serotype 1 strain, and a hemolytically less active HlyII, requiring high concentrations of Ca<sup>2+</sup> for hemolytic activity but not inducible by  $Ca^{2+}$ , was detected in serotype 2 (8, 10).

Cloning and expression of the structural gene (hlyIA) for the 105-kDa HlyI protein in *Escherichia coli* revealed that HlyI is synthesized in the form of an inactive prohemolysin (HlyIA) which could be activated in *trans* by complementation with the *E. coli* or *Proteus vulgaris* activator gene hlyC; secretion could be achieved by complementation with the *E. coli* hlyBD secretion genes (13). These experiments show that HlyI is strongly related to the *E. coli* alpha-hemolysin and the *P. vulgaris* hemolysin and predict a similar genetic organization for the *A. pleuropneumoniae* hemolysin I determinant. Chang et al. (4) have cloned the structural gene appA and its activator gene appC of the hemolysin from an A. pleuropneumoniae serotype 5 strain, using as a gene probe the genes lktA and lktC, which encode the Pasteurella haemolytica leukotoxin. Their sequence analysis of appA and appC revealed homology to both the E. coli alphahemolysin and the leukotoxin (4). The DNA sequences of a structural hemolysin gene and its activator gene from A. pleuropneumoniae serotype 9 have been determined and show almost complete identity to the analog genes appA and appC from serotype 5 (36a). The deduced restriction map for the structural hemolysin gene appA from serotype 5, however, showed strong differences from that of the hlyIA from serotype 1 isolated by Gygi et al. (13).

We report here the nucleotide sequence of the gene encoding HlyI in A. pleuropneumoniae serotype 1 type strain 4074 (36) and show the binding of  $^{45}Ca^{2+}$  to HlyI as well as to prohemolysin HlyIA. From comparison of DNA sequences and deduced amino acid sequences with those of related hemolysins and cytotoxins, we conclude that at least two types of hemolysins are produced by this species. One is HlyI, resembling the *E. coli* alpha-hemolysin. The second is App5, which was cloned from serotypes 5 and 9 (4, 36a) and shows closer similarities to the leukotoxin of *Pasteurella* haemolytica.

## MATERIALS AND METHODS

**Bacterial strains, plasmids, and growth conditions.** The following bacterial strains were used: A. pleuropneumoniae serotype 1 type strain 4074 (29); A. pleuropneumoniae serotype 3 reference strain S1421 (10); and E. coli JF586, a DHI (33) derivative which was made resistant to streptomycin with the following genotype: recA1 endA1 gyrA96 thi-1 hsdR17 hsdM<sup>+</sup> supE44 F<sup>-</sup> lambda<sup>-</sup> rpsL20 Sm<sup>r</sup> (received

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from P. Prentki, University of Geneva, Geneva, Switzerland). E. coli XL1-blue endA hsdR17 supE44 lambda<sup>-</sup> recA1 del(proAB-lac) [F' proAB lacI<sup>q</sup>ZdelM15 Tn10 (Tc<sup>r</sup>)] (3) was used for cloning with pBluescript plasmid. E. coli 5K thi-1 thr-1 leu-6 supE44 lacY1 tonA21 hsdR<sup>-</sup>S<sup>+</sup> was used as host for the production of plasmid-encoded E. coli alpha-hemolysin (pEK50) and recombinant A. pleuropneumoniae HlyI (pJFF702).

Plasmid pJFF702 containing the A. pleuropneumoniae hlyIA gene was described by Gygi et al. (13). Plasmid pLG575 (26) contains the E. coli secretion genes for alphahemolysin hlyBD and was used in strain 5K for the secretion of recombinant HlyIA. Plasmid pEK50 (17) was used for the production of E. coli alpha-hemolysin. Plasmid pBluescriptII SK<sup>-</sup> (Stratagene, La Jolla, Calif.) was used for subcloning. Plasmid pAP13 (30), which is a pBR322 derivative containing an IS1 element and an  $rpsL^+$  gene (obtained from P. Prentki), was used for the formation of deletion derivatives and subsequent DNA analysis of the hlyIA gene.

*E. coli* strains were grown in Luria-Bertani broth (33). Drugs used were ampicillin at 25 µg/ml, tetracycline at 25 µg/ml, chloramphenicol at 25 µg/ml, and streptomycin at 500 µg/ml. *A. pleuropneumoniae* was grown in Columbia broth (BBL Microbiology Systems, Cockeysville, Md.) supplemented with 1% IsoVitaleX (BBL) and 10 µg of  $\beta$ -NAD (Sigma Chemical Co., St. Louis, Mo.) per ml.

DNA sequencing and analysis. DNA sequencing was performed by the dideoxy chain-termination method (34) utilizing [<sup>35</sup>S]dATP (Amersham International plc, Amersham, Buckinghamshire, United Kingdom) and T<sub>7</sub> DNA polymerase with the sequencing kit Sequenase (United States Biochemical, Cleveland, Ohio) for the direct sequencing of double-stranded plasmid DNA (42). GC-rich segments were also sequenced by using the dGTP-analog dITP. The following oligonucleotide primers were used for sequencing: T<sub>3</sub> primer (Stratagene), 5'-ATTAACCCTCACTAAAG-3'; primer G258, corresponding to the left-end sequence of IS1, 5'-AG CCACTGGAGCACCTC-3'; primer Pr-1, 5'-TCAGTAACT TATCAAAC-3'; primer Pr-2, 5'-ATCACCGGTAAATGC AC-3'; primer Pr-3, 5'-TTATCAACGATTTTTTC-3'; Pr-4, 5'-TCAATAATGATATGACG-3'; and primer Pr-5, 5'-TCA CCATCACCGCCATT-3'. The oligonucleotides Pr-1 to Pr-5, corresponding to HlyIA sequences and G258, were synthesized on an Applied Biosystems 380A DNA Synthesizer (Applied Biosystems, Foster City, Calif.). The DNA sequence was assembled from the individual sequences and analyzed by using the PC-Gene DNA and protein analysis programs (University of Geneva). Homology estimations were done by the method of Myers and Miller (27), and RNA secondary-structure calculations were made by the method of Tinoco et al. (40).

<sup>45</sup>Ca<sup>2+</sup>-binding assay. Pure HlyI was obtained as described earlier (9). Crude HlyI from *A. pleuropneumoniae* serotype 1 strain and crude *E. coli* alpha-hemolysin from strain 5K harboring plasmid pEK50 was obtained from supernatants of cultures in mid-exponential growth phase followed by 1,000× concentration with 60% ammonium sulfate precipitation and subsequent purification on a Sephacryl S200 column mainly to remove medium components. Supernatant from *A. pleuropneumoniae* serotype 3, which does not show measurable hemolytic activity, was treated identically. Recombinant *A. pleuropneumoniae* prohemolysin HlyIA was obtained from *E. coli* 5K harboring the plasmids pJFF702 (Ap<sup>r</sup>) and pLG575 (Cm<sup>r</sup>). The strain was grown in Luria-Bertani medium to an  $A_{650}$  of 0.5. Induction of the vector's *lacZ* promoter on pJFF702 was obtained by

the addition of 0.1 mM (final concentration) isopropyl-B-Dthiogalactosidase (IPTG) and subsequent growth for a further 3 hours. The supernatant of the culture was then concentrated and purified as described above. As a negative control, an isogenic E. coli strain which did not contain the hlyIA gene was treated identically. Samples of the concentrated supernatants from the various strains containing 40 µg of proteins or 0.8 µg of pure HlyI (for the immunoblot, 0.2  $\mu$ g of HlyI) were separated on a 10% polyacrylamide gel (20) and transferred by electrotransfer (33) to nitrocellulose filters (BioRad, Richmond, Calif.). Incubation with <sup>45</sup>Ca<sup>2+</sup> was performed as described by Boehm et al. (2). The Ca<sup>2+</sup>-binding proteins were visualized by autoradiography. The presence of HlyI or HlyIA was visualized by an immunoblot technique using polyclonal anti-HlyI antibodies as described earlier (13).

Formation of series of deletion derivatives of hlyIA. Deletions formed by transposition events of the resident ISI sequence on plasmid pAP13 into the cloned hlyIA gene were obtained by selecting for the loss of the streptomycin sensitivity gene  $rpsL20^+$  on plates containing 500 µg of streptomycin per ml. Plasmids isolated from the selected clones were analyzed by restriction enzyme mapping in order to determine the extent of the deletion. All other in vitro manipulations of DNA-like restriction enzyme digestions, DNA ligations, gel electrophoresis analysis, and transformation were done according the protocols of Sambrook et al. (33).

**DNA sequence accession number.** The GenBank-EMBL accession number for primary nucleotide sequence data of the presented sequence is X52899.

#### RESULTS

DNA sequence analysis of hlyIA. The 3.6-kb PvuII-EcoRI fragment from plasmid pJFF702 (13) containing hlyIA and flanking sequences from the hemolysin determinant of A. pleuropneumoniae serotype 1 was subcloned into plasmid pAP13. A series of 44 plasmid derivatives which contained successive deletions with an interval of approximately 100 bp from the C-terminal end into the hlyIA gene were selected and used for subsequent DNA sequence analysis. DNA sequence analyses of the deletion derivatives were made by using the G258 primer, which corresponds to the left end of IS1. A 500-bp segment of hlyIA into which an IS1-induced deletion could not be inserted was sequenced by using oligonucleotide primers P-1 to P-5, which were synthesized successively according to the determined DNA sequence. The sequence strategy was such that each segment was sequenced at least twice, and most segments were sequenced three times. Figure 1 shows the complete assembled sequence of the sense strand of hlyIA and the flanking segments. The DNA sequence was screened for sequences which resembled the consensus sequences for E. coli ribosome-binding sites (Shine-Dalgarno sequences) (35), promoter sequences, transcription stop signals, and open reading frames (ORFs). The DNA sequence contains a large ORF of 3,072 bp starting at coordinate 58 with ATG and terminating at coordinate 3129 with the stop codon TAA. This ORF, which encodes a protein of 1,023 amino acids with a predicted molecular mass of 110.1 kDa, is preceded by a consensus sequence for a putative ribosome-binding site (35) 5 bp upstream of the ATG initiation codon. The 110.1-kDa polypeptide coincides in size with HlyI protein, which has an apparent size of 105 kDa on sodium dodecyl sulfate-polyacrylamide gel electrophoresis, suggesting that

60 l	1090	1100	1110	1120	1130	1140
NET	TTCGGTTATGAAG PheGlyTyrGluG	GTGATAGTTT lyAspSerLe	ATTAGCTTCA uLeuAlaSer	TTCTACCGTG PheTyrArgG	AAACCGGTGC luThrGlyAl	GATTGAA alleGlu
120 	1150	1160	1170	1180	1190 	1200
AAAA Lys	GCGGCATTAACCA AlaAlaLeuThr7	CGATTAACAG	TGTGTTAAGT rValLeuSer	GCGCGTTCCG AlaArgSerA	CAGGTGTTGG laGlyValGl	GGCTGCT YAlaAla
180	1210	1220	1230	1240	1250	1260
AGCÀ hAla	GCAACCGGCTCAT AlaThrGlySerI	TAGTCGGTGC LeuValGlyAl	GCCGGTAGCA AProValAla	GCTTTAGTTA	GTGCAATCAC erAlaIleTh	CGGTATT rGlyIle
240	1270	1280	1290	1300	1310	1320
rCTT rLeu	ATTTCAGGTATT IleSerGlyIleI	TAGATGCTTC LeuAspAlaSe	TAAACAGGCA	ATCTTCGAAC	GAGTTGCAAC rgValAlaTh	GAAATTA
300 I	1330	1340	1350	1360	1370	1380
AAAĊ sAsn	GCGAATAAGATT	GACGAATGGGA AspGluTrpGl	GAAAAAAACAC .uLysLysHis	GGTAAAAACT GlyLysAsnT	ATTTTGÅAAA yrPheGluAs	CGGTTAT
360	1390	1400	1410	1420	1430	1440
GGAA rGlu	GACGCCCGCCAT	ICCGCATTCTI	AGAAGATACO	TTTGAATTGT	I TATCACAATA	CAATAAA
420	1450	1460	1470	1480	1490	1500
 ATTA sLeu	GAGTATTCGGTA	AGCGTGTCGT	TGCTATTACO	CAACAGCGTT	GGGATGTCAA	TATCGGI
480	GIUTYTSETVAL	1520	1530	GINGINARGT	rpAspValAs	nileGly
AACG	NaeI GAACTTGCCGGC	ATTACTCGCAA	AGGTTCTGAT		HindIII GTAAAGCTTA	
nThr	GluLeuAlaGly	[leThrArgLy	sGlySerAsp	ThrLysSerG	lyLysAlaTy	rValAsp
540   TAGC	1570 	1580 	1590 	1600 	1610 	1620 
pSer	TTCTTTGAAGAAG PhePheGluGluG	GGAAAACTITI GlyLysLeuLe	AGAGAAAAGAA uGluLysGlu	ACCGGATCGTT IProAspArgP	TTGATAAAAA heAspLysLy	AGTGTTT sValPhe
600 	1630	1640	1650	1660	1670	1680
AGGT aGly	GATCCGCTTGAA AspProLeuGlu	GGTAAAATCGA GlyLysIleAs	CCTTTCTTC pLeuSerSer	ATTAACAAAA IleAsnLysT	CCACTTTATT hrThrLeuLe	GAAATTI
660	1690	1700	1710	1720	1730	1740
TGCG pAla	GTTACGCCGGTC	TTTACCGCAGG	TGAAGAGATT	CGTGAGCGTA	AGCAAACCGG	ا TAAATAC vLvsTvt
720	1750	1760	1770	1780	1790	1800
TTTA	CAATATATGACC	GAATTATTCGI	TAAAGGTAA	GAAAAATGGG	BstEII TGGTAACCGG	TGTGCAC
780	GInTyrMetThr	1820	1830	SGluLysTrpV	alValThrGl	yValGlr 1860
CGTA	TCACATAATGCG	I ATTTATGACT	TACGAATCT	TATCCAATTAG	CGATAGATAA	AAAAGGI
aVal	SerHisAsnAla	IleTyrAspTy	rThrAsnLeu	lleGlnLeuA	laIleAspLy	sLysGly
840   TGCA				1900   CCTCACAAAA	1910   אדכאדככדאר	1920
pAla	GluLysArgGln	ValThrIleG	luSerHisLeu	IGlyGluLysA	snAspArgI1	eTyrLeu
900 I	1930 	1940 	1950 	1960 	1970 	1980 
TAAA YLys	TCATCCGGTTCA SerSerGlySer	TCTATCGTATA SerIleValTy	ATGCGGGTAAC /rAlaGlyAsi	CGGACATGATG nGlyHisAspV	TAGCATATTA alalaTyrTy	CGATAAA
960	1990 I	2000	2010	2020 SnoT	2030	2040
GGCÅ aAla	ACCGATACAGGT ThrAspThrGly	TACTTAACATT TyrLeuThrPi	TTGACGGÁCA heAspGlyGli	AA <u>GTGCAC</u> AGA nSerAlaGlnL	AAGCCGGTGA ysAlaGlyGl	ATATATI uTyrIle
1020	2050 I	2060 I	2070 I	2080	2090 I	2100
AAAT uAsn	GTCACTAAAGAA ValThrLysGlu	CTTAAAGCTG LeuLysAlaAs	ATGTAAAAGT spValLysVal	TTTAAAAGAAG lLeuLysGluV	TGGTTAAAAC alValLysTh	TCAGGA
1080	2110	2120	2130	2140	2150	2160
i AAAG SLys	I ATTTCAGTTGGA IleSerValGly	 AAAACGTGCAC LysThrCysSe	J TGAAAAATTI CGluLysLev	 AGAATATCGTG 1GluTyrArgA	ATTATGAGTI SpTyrGluLe	TAAGCCCA
	-			-		

 10
 20
 30
 40
 50
 60

 I
 I
 I
 I
 --SD-- I

 GAGCTAAAAAATAAACAAAATTTTTAAATTTTCATTAGTAAATAGCTA<u>AGGAGA</u>CAACATG
 MET
 MET
 MET

 ${\tt AlaAsnSerGlnLeuAspArgValLysGlyLeuIleAspSerLeuAsnGlnHisThrLyster and a start of the start of$ NaeI AGTGCAGCTAAATCAGGT<u>GCCGGC</u>GCATTAAAAAATGGTTTGGGACAGGTGAAGCAAGC SerAlaAlaLysSerGlyAlaGlyAlaLeuLysAsnGlyLeuGlyGlnValLysGlnAl GGGCAGAAATTAATTTTATATATTCCGAAAGATTATCAAGCTAGTACCGGCTCAAGTCT  ${\tt GlyGlnLysLeuIleLeuTyrIleProLysAspTyrGlnAlaSerThrGlySerSerLeuTyrIleProLysAspTyrGlnAlaSerThrGlySerSerLeuTyrIleProLysAspTyrGlnAlaSerThrGlySerSerLeuTyrIleProLysAspTyrGlnAlaSerThrGlySerSerLeuTyrIleProLysAspTyrGlnAlaSerThrGlySerSerLeuTyrIleProLysAspTyrGlnAlaSerThrGlySerSerLeuTyrIleProLysAspTyrGlnAlaSerThrGlySerSerLeuTyrIleProLysAspTyrGlnAlaSerThrGlySerSerLeuTyrIleProLysAspTyrGlnAlaSerThrGlySerSerLeuTyrIleProLysAspTyrGlnAlaSerThrGlySerSerLeuTyrIleProLysAspTyrGlnAlaSerThrGlySerSerLeuTyrIleProLysAspTyrGlnAlaSerThrGlySerSerLeuTyrIleProLysAspTyrGlnAlaSerThrGlySerSerLeuTyrIleProLysAspTyrGlnAlaSerThrGlySerSerLeuTyrIleProLysAspTyrGlnAlaSerThrGlySerSerLeuTyrIleProLysAspTyrGlnAlaSerThrGlySerSerLeuTyrIleProLysAspTyrGlnAlaSerThrGlySerSerLeuTyrIleProLysAspTyrGlnAlaSerThrGlySerSerLeuTyrIleProLysAspTyrGlnAlaSerThrGlySerSerLeuTyrIleProLysAspTyrGlnAlaSerThrGlySerSerLeuTyrIleProLysAspTyrGlnAlaSerThrGlySerSerLeuTyrIleProLysAspTyrGlnAlaSerThrGlySerSerLeuTyrIleProLysAspTyrGlnAlaSerThrGlySerSerLeuTyrIleProLysAspTyrGlnAlaSerThrGlySerSerLeuTyrIleProLysAspTyrGlnAlaSerThrGlySerSerLeuTyrIleProLysAspTyrGlnAlaSerThrGlySerSerLeuTyrIleProLysAspTyrGlnAlaSerThrGlySerSerLeuTyrIleProLysAspTyrGlnAlaSerThrGlySerSerLeuTyrIleProLysAspTyrGlnAlaSerThrGlySerSerLeuTyrIleProLysAspTyrGlnAlaSerThrGlySerSerLeuTyrIleProLysAspTyrGlnAlaSerThrGlySerSerLeuTyrIleProLysAspTyrGlnAlaSerThrGlySerSerLeuTyrIleProLysAspTyrGlnAlaSerThrGlySerSerLeuTyrIleProLysAspTyrGlnAlaSerThrGlySerSerLeuTyrIleProLysAspTyrGlnAlaSerThrGlySerSerLeuTyrIleProLysAspTyrGlnAlaSerThrGlySerSerLeuTyrIleProLysAspTyrGlnAlaSerThrGlySerThrGlySerSerLeuTyrIleProLysAspTyrGlnAlaSerThrGlySerThrGlySerSerLeuTyrIleProLysAspTyrGlnAlaSerThrGlySerThrGlySerThrGlySerThrGlySerThrGlySerThrGlySerThrGlySerThrGlySerThrGlySerThrGlySerThrGlySerThrGlySerThrGlySerThrGlySerThrGlySerThrGlySerThrGlySerThrGlySerThrGlySerThrGlySerThrGlySerThrGlySerThrGlySerThrGlySerThrGlySerThrGlySerThrGlySerThrGlySerThrGlySerThrGlySerThrGlySerThrGlySerThrGlySerThrGlySerThrGlySerThrGlySerThrGlySerThrGlySerThrGlySerThrGlySerThrGlySerThrGlySerThrG$ AATGATTTAGTGAAAGCGGCGGAGGCTTTAGGGATCGAAGTACATCGCTCGGAAAAAAA AsnAspLeuValLysAlaAlaGluAlaLeuGlyIleGluValHisArgSerGluLysAs KpnI GGTACCGCACTAGCGAAAGAATTATTCGGTACAACGGAAAAACTATTAGGTTTCTCGGA GlyThrAlaLeuAlaLysGluLeuPheGlyThrThrGluLysLeuLeuGlyPheSerGl CGAGGCATCGCATTATTTGCACCTCAGTTTGATAAGTTACTGAATAAGAACCAAAAATT ArgGlyIleAlaLeuPheAlaProGlnPheAspLysLeuLeuAsnLysAsnGlnLysLe SerLysSerLeuGlyGlySerSerGluAlaLeuGlyGlnArgLeuAsnLysThrGlnTh GCACTTTCAGCCTTACAAAGTTTCTTAGGTACGGCTATTGCGGGGTATGGATCTTGATAG 
 Image: State GTGGATCTAGCCGCTCAGTTAGTGGATAACATTGCAAGTGCAACGGGTACGGTGGATGC ValAspLeuAlaAlaGlnLeuValAspAsnIleAlaSerAlaThrGlyThrValAspAl TTTGCCGAACAATTAGGTAAATTGGCAATGCCTTATCTAACACTCGCATTAAGCGGTTT PheAlaGluGlnLeuGlyLysLeuAlaMetProTyrLeuThrLeuAlaLeuSerGlyLe DraII GCAAGTAAGTTAAATAACCTTCCAGATTTAAGCCTTGCAGGACCTGGGGTTTGATGCCGT AlaSerLysLeuAsnAsnLeuProAspLeuSerLeuAlaGlyProGlyPheAspAlaVa TCAGGTATCTTATCTGTTGTTTCGGCTTCATTCATTTTAAGTAATAAAGATGCCGATG SerGlyIleLeuSerValValSerAlaSerPheIleLeuSerAsnLysAspAlaAspAl GGTACAAAAGCGGCGGCAGGTATTGAAATCTCAACTAAAATCTTAGGCAATATCGGTAA GlyThrLysAlaAlaAlaGlyIleGluIleSerThrLysIleLeuGlyAsnIleGlyLy GCGGTTTCTCAATATTATTGCGCAACGTGTGGCGGCAGGCTTATCCACAACTGCGGC AlavalserglntyrileilealaglnargvalalaalaglyLeuserthrthralaal ACCGGTGGTTTAATCGGTTCGGTCGTAGCATTAGCGATTAGCCCGCTTTCGTTCTTAAA ThrGlyGlyLeuIleGlySerValValAlaLeuAlaIleSerProLeuSerPheLeuAs Sspl GTTGCGGATAAGTTTGAACGTGCGAAACAGCTTGAACAATATTCGGAGCGCTTTAAAAA ValalaaspLysPheGluargAlaLysGlnLeuGluGlnTyrSerGluArgPheLysLy

FIG. 1. DNA sequence of *hlyIA* gene and flanking segments from *A. pleuropneumoniae* serotype 1 strain 4074. The sites for some restriction enzymes are shown. The putative ribosome-binding sites are indicated by --SD--. A consensus sequence for a promoter is indicated by <-35> and <-10>. Symbols: —, three hydrophobic domains;  $\blacksquare$ , glycine-rich repeated sequences;  $\blacksquare$ , beginning of the ORF of the putative *hlyIB* gene.

 TABLE 1. Homologies of hlyIA from A. pleuropneumoniae

 serotype 1 to related hemolysins and cytotoxins

2170	2180	2190	2200	2210	2220
TTCGAACTTGGGA	ACGGTATCAG	AGCTAAAGAT	GAATTACATT	CTGTTGAAGA	AATTATC
PheGluLeuGIYA	sngiyilear	galaLysAsp	GIULEUHISS	ervalgiugi	
2230 	2240	2250	2260	22/0   NC	2280 0I
GGTAGTAATCGTA GlySerAsnArgL	AAGACAAATT ysAspLysPh	CTTTGGTAGT ePheGlySer	CGCTTTACCG ArgPheThrA	ATATTTT <u>CCA</u> spilePheHi	<u>TGG</u> TGCG SGlyAla
2290	2300	2310	2320	2330	2340
AAAGGCGATGATG	 AAATCTACGG	 TAATGACGGC	ECORV CACGATATCT	 TATACGGAGA	CGACGGT
LysGlyAspAspG	luIleTyrGl	yAsnAspGly	HisAspIleL	euTyrGlyAs	pAspGly
2350	2360	2370	2380	2390	2400
AATGATGTAATCC	ATGGCGGTGA	CGGTAACGAC	CATCTTGTTG	GTGGTAACGG	AAACGAC
ASIASPValler	2420	2420		2450	2460
2410	2420		2440	2450	2460
CGATTAATCGGCG ArgLeuIleGlyG	GAAAAGGTAA lyLysGlyAs	nAsnPheLeu	AATGGCGGTG AsnGlyGlyA	ATGGTGACGA .spGlyAspAs	TGAGTTG pGluLeu
2470	2480	2490	2500	2510	2520
CAGGTCTTTGAGG	 GTCAATACAA	CGTATTATTA	 .GGTGGTGCGG	 GTAATGACAT	TCTGTAT
GlnValPheGluG	lyGlnTyrAs	nValLeuLeu	GlyGlyAlaG	lyAsnAspIl	eLeuTyr
2530	2540	2550	2560	2570	2580
GGCAGCGATGGTA	CTAACTTATI	TGACGGTGGT	GTAGGCAATG	ACAAAATCTA	CGGTGGT
GIYSERASpGIYI	nrasnleupn	easpgiygiy	Valgiyasha		rgiygiy
2590	2600 	2610 	2620	2630	2640
TTAGGTAAGGATA LeuGlyLysAspI	TTTATCGCTA leTyrArgTy	CAGTAAGGAG rSerLysGlu	TACGGTCGTC TyrGlyArgH	ATATCATTAT	TGAGAAA eGluLys
2650	2660	2670	2680	2690	2700
 GGCGGTGATGATG	 ATACGTTATT	 GTTATCGGAT	 CTTAGTTTTA	 AAGATGTAGG	 ATTTATC
GlyGlyAspAspA	spThrLeuLe	uLeuSerAsp	LeuSerPheL	ysAspValGl	yPheIle
2710	2720	2730	2740	2750	2760
AGAATCGGTGATG	ATCTTCTTGT	GAATAAAAGA	ATCGGAGGAA	CACTGTATTA	CCATGAA
ArgileGlyAspA	spLeuLeuVa	lAsnLysArg	IleGlyGlyT	nrLeuTyrTy	rHisGlu
2770 	2780 	2790 	2800 	2810 	2820 I
GATTACAATGGGA AspTyrAsnGlyA	ATGCGCTCAC	GATTAAAGAT	TGGTTCAAGG TrpPheLysG	AAGGTAAAGA luGlyLysGl	AGGACAA uGlyGln
2830	2840	2850	2860	2870	- 2880
			GGAGCTTATG		 ATTATICTIC
AsnAsnLysIle	luLysIleVa	lAspLysAsp	GlyAlaTyrV	alLeuSerGl	nTyrLeu
2890	2900	2910	2920	2930	2940
ACTGAACTGACAG	CTCCTGGAAG	 AGGTATCAA1	TACTTTAATG	 GGTTAGAAGA	 AAAATTG
ThrGluLeuThr#	laProGlyAr	gGlyIleAsn	TyrPheAsnG	lyLeuGluGl	uLysLeu
2950	2960	2970 I	2980 I	2990 I	3000
TATTATGGAGAAG	GATATAATGC	ACTTCCTCAA	CTCAGAAAAG	ATATTGAACA	AATCATT
3010	3020	3030	3040	3050	3060
		BclI			
SerSerThrGly	laPheThrGl	YASPHisGly	LysValSerV	alGlySerGl	YGIYPro
3070	3080	3090	3100	3110	3120
 TTAGTCTATAAT?	ACTCAGCTAA	 CAATGTAGCA	 AATTCTTTGA	 GTTATTCTTI	AGCACAA
LeuValTyrAsnA	AsnSerAlaAs	mAsnValAla	AsnSerLeuS	erTyrSerLe	uAlaGln
3130	3140	3150	3160	3170	3180
GCAGCTTAAGAT	GTTATTTTA	GATGATAAAI	AGCAATCCTA	TATATATTAG	GTGTGTA
***BIABIA		<			
3190 . l	3200  S	3210 D	3220	3230 	3240 
GGATTGCTATTTT	ATTTATGGAG	GAGCAAATGG MET	ATTTTTATCG	GGAAGAAGAC	TACGGAT
	FI	G. 1—Con	tinued.		

Gene <sup>a</sup>		% Homology (DNA/amino acid) to:				
	hlyIA	appA	hlyA	lktA		
hlyIA	100/100	50.7/42.7	61.9/56.4	52.2/41.1		
appA	50.7/42.7	100/100	53.3/42.6	66.6/66.2		
hlvA	61.9/56.4	53.3/42.6	100/100	52.5/44.0		
lktA	52.2/41.1	66.6/66.2	52.5/44.0	100/100		

<sup>a</sup> The following structural genes of cytotoxins were compared: hlyIA, encoding HlyI of A. pleuropneumoniae serotype 1 type strain 4074 (this paper); appA, encoding the hemolysin of A. pleuropneumoniae serotype 5 strain (4); hlyA, encoding the E. coli alpha-hemolysin (6, 14); and lktA, encoding the leukotoxin of Pasteurella haemolytica Al. (22).

this ORF represents *hlyIA*, the structural gene for HlyI. The nucleotide sequence upstream of *hlyIA* shows no consensuslike promoter sequence but contains the C-terminal end of an ORF that shows high homology with the *E. coli hlyC* gene (6, 14). A sequence that can encode an mRNA structure very similar to the rho-independent transcription signal of *E. coli* (31) was identified downstream of *hlyIA* (Fig. 1). This structure consists of a 19-bp stem and a 5-base loop region followed by a T-rich stretch. The calculated stability of the RNA secondary structure has a  $\Delta G$  value of -87.4 KJ/mol. Further downstream, the beginning of an ORF with a consensus sequence for a ribosome-binding site is found. This sequence shows strong homology to the *E. coli hlyB* hemolysin secretion gene (6).

Comparisons of the DNA sequence of hlyIA and the derived amino acid sequence with those of the E. coli alpha-hemolysin hlyA, A. pleuropneumoniae hemolysin serotype 5 appA, and Pasteurella haemolytica leukotoxin lktA are shown in Table 1. They reveal that hlyIA has a strong homology to hlyA and a lesser homology to appA and lktA at both the DNA and amino acid levels. In addition, hlyIA shows a relatively high homology (56% on the DNA level and 52.2% on the amino acid level) with the leukotoxin of Actinobacillus actinomycetemcomitans (18, 21). Further analysis of the derived amino acid sequence of the 110.1-kDa ORF of *hlyIA* revealed features that are similar to structures found in other hemolysins and cytotoxins of the RTX (repeats in the structural toxin) family (38). Three strong hydrophobic regions are found in the N-terminal third of the molecule, and 13 glycine-rich repeated sequences corresponding to the consensus sequence L/V-X-G-G-X-G-N/D-D-X that occurs in the E. coli alpha-hemolysin (2, 7, 23) are found in the C-terminal third of the sequence (Fig. 1). Alignment of the hydropathy profiles of HlyIA with that of HlyA, AppA, and LktA (results not shown) indicated that the three hydrophobic domains of all three cytotoxins were congruent. Comparing the amino acid sequences of the three hydrophobic domains of HlvIA with those of the other three cytotoxins, we detected high conservation of 75% homology between HlyIA and HlyA (sequence taken from reference 6) and similarly between AppA and LktA (sequences taken from references 4 and 22), while significantly lower homologies of 50% were determined between HlyIA and AppA, HlyI and AppA, HlyIA and LktA, and HlyA and LktA. At both the N terminus and the C terminus, HlyIA shows high primary sequence divergence from HlyA, AppA, and LktA.

The location of the 13 glycine-rich repeated domains on HlyIA is congruent with those on the E. coli HlyA (7) and corresponds with the location of the nine glycine-rich re-

TABLE 2. Glycine-rich repeated domains<sup>a</sup>

Repeat	HlyIA	HlyA	АррА	LktA
1	IIGSNRKDK	LIGTTRADK	IIGSQFNDI	IIGTSHNDI
2	FFGSRFTDI	FFGSKFADI	FKGSQFDDV	FKGSLFMDA
3	FHGAKGDDE	FHGADGDDH	FHGGNGVDT	FNGGDGVDT
4	IYGNDGNDI	IEGNDGNDR	IDGNDGDDH	IYGNDGNDR
5	LYGGDGNDV	LYGDKGNDT	LFGGAGDDV	LFGGKGDDI
6	IHGGDGNDH	LSGGNGDDQ	IDGGNGNNF	LDGGNGDDF
7	LVGGNGNDR	LYGGDGNDK	LVGGTGNDI	IDGGKGNDL
8	LIGGKGNNF	LIGGAGGNY	ISGGKDNDI	LHGGKGDDI
9	LNGGDGDDE	LNGGDGDDE		
10	LLGGAGNDI	LSGGKGNDK		
11	LYGSDGTNL	LYGSEGADL		
12	FDGGVGNDK	LDGGEGNDL		
13	IYGGLGKDI	LKGGYGNDI		

<sup>*a*</sup> According to the *E. coli* alpha-hemolysin consensus sequence (L/V-X-G-G-X-G-N/D-D-X) (2, 7, 23) of HlyIA and the structural proteins of related hemolysins and cytotoxins. The amino acid sequences were taken from the following sources: *A. pleuropneumoniae* serotype 1 hemolysin (HlyIA) (our results), *A. pleuropneumoniae* serotype 5 hemolysin (AppA) (4), *E. coli* alpha-hemolysin (HlyA) (6), and *Pasteurella haemolytica* leukotoxin (LktA) (22).

peats on AppA and LktA. Table 2 gives a comparative summary of the repeated domains of the four cytotoxins.

Ca<sup>2+</sup> binding of HlyIA. The capability of HlyI to bind Ca<sup>2+</sup> was assayed on calcium blots as described by Boehm et al. (2), using  ${}^{45}Ca^{2+}$ . Autoradiographs showed that  $1,000 \times$ concentrated supernatants from A. pleuropneumoniae serotype 1 or from an alpha-hemolysin-producing E. coli strain showed a protein in the 110-kDa range that was able to bind Ca<sup>2+</sup>, while the other proteins found in the supernatant did not bind Ca<sup>2+</sup>. Supernatant of the A. pleuropneumoniae serotype 3 reference strain, which does not show hemolytic activity, had no Ca<sup>2+</sup>-binding protein (results not shown). In order to verify that HlyI was the Ca<sup>2+</sup>-binding protein, we used concentrated supernatant from a recombinant E. coli strain which produces A. pleuropneumoniae prohemolysin HlyIA and showed that the recombinant 105-kDa protein binds  ${}^{45}Ca^{2+}$  (Fig. 2). An isogenic *E. coli* strain that did not contain the cloned hlyIA gene which was used as a negative control showed no  $Ca^{2+}$  binding (Fig. 2). In addition, Fig. 2 shows that purified HlyI from A. pleuropneumoniae binds Ca<sup>2+</sup>, demonstrating that both pure HlyI and recombinant HlyIA are able to bind  $Ca^{2+}$ .

### DISCUSSION

DNA sequence analysis of the cloned DNA fragment containing the structural HlyI gene, hlyIA, from A. pleuropneumoniae serotype 1 strain 4074 revealed an ORF coding for a protein of 1,023 amino acids with a calculated molecular size of 110.1 kDa, corresponding to HlyIA. This ORF is preceeded by a consensus sequence for a ribosome-binding site 5 bp upstream of the translation initiation codon, AUG. It shows high homology at both the nucleotide and amino acid sequence levels to the E. coli hlyA gene and to a lesser extent to the Pasteurella haemolytica leukotoxin lktA and the A. pleuropneumoniae serotype 5 appA genes. Subcloning experiments indicated that the coding sequence for HlyIA must begin upstream of the NaeI site at coordinate 140 (results not shown). Comparison of the sequences upstream and downstream of hlyIA with that of the hemolysin determinant of the E. coli alpha-hemolysin (6, 14) indicates that the sequence upstream of hlyIA shows homology to the C-terminal part of the E. coli hemolysin activator gene hlyC



FIG. 2. <sup>45</sup>Ca<sup>2+</sup> binding of HlyI and HlyIA. (A) <sup>45</sup>Ca<sup>2+</sup> blot; (B) anti-HlyI immunoblot of proteins that were separated on a 10% sodium dodecyl sulfate-polyacrylamide gel and subsequently transferred to nitrocellulose. Lane 1, purified HlyI from *A. pleuropneumoniae* (0.8  $\mu$ g for <sup>45</sup>Ca<sup>2+</sup> blot and 0.2  $\mu$ g for immunoblot); lane 2, proteins, including recombinant HlyIA of *E. coli* XL1-blue containing plasmids LG757 and pJFF702 (*hlyIA*); lane 3, *E. coli* XL1-blue containing plasmid LG757 (negative control); lane S, prestained molecular mass protein standards (BioRad; product 161-0305) with apparent molecular masses of 106, 84, and 47 kDa. These prestained protein standards bind <sup>45</sup>Ca<sup>2+</sup> and therefore are most useful on <sup>45</sup>Ca<sup>2+</sup> blots.

and suggests the presence of the A. pleuropneumoniae analog hlyIC. The sequences downstream of hlyIA shows high homology to the N terminus of the E. coli hemolysin secretion gene hlyB and are suggested as being the beginning of the analog gene hlyIB. Both an activator gene and secretion genes have been previously suggested by complementation experiments (13) to be present in A. pleuropneu*moniae* serotype 1. They have also been found in a serotype 5 strain (4). Between the hlyIA and the hlyIB genes, a putative rho-independent transcription termination signal was detected (Fig. 1). The calculated stability of the secondary structure of the RNA from this sequence ( $\Delta G = -87.4$ KJ/mol) is somewhat weaker than that of similar sequences found in the E. coli hemolysin determinant between hlyA and *hlyB* (calculated  $\Delta G = -125.7$  KJ/mol) (6) and in *Pasteurella* haemolytica between lktA and lktB (calculated  $\Delta G = -101.2$ JK/mol) (38), which are known to regulate the expression of the genes involved in hemolysin and leukotoxin secretion (hlyBD and lktAB, respectively) (6, 15, 39). We therefore assume that the transcription termination signal between hlyIA and hlyIB has a similar function in A. pleuropneumo*niae* in the regulation of expression of the secretion gene(s).

Further analysis of the derived amino acid sequence of HlyIA showed that this molecule contains the typical features of the RTX toxin family (38), namely, three hydrophobic domains and 13 glycine-rich repeats (37) (Fig. 1; Table 2). Comparison with the sequences of HlyA, AppA, and LktA revealed the interesting fact that a much higher similarity of HlyIA is found with the strongly hemolytic E. coli HlyA than with the weakly hemolytic Pasteurella haemolytica leukotoxin LktA or with the A. pleuropneumoniae serotype 5 hemolysin AppA. This is true of the total sequence and especially of the hydrophobic domains and the glycine-rich repeats. Judging from the sequence data, it is most likely that at least two types of hemolysins, one resembling HlyA and another resembling LktA, are found in A. pleuropneumoniae strains. The resemblance of HlyIA to the leukotoxin of A. actinomycetemcomitans lies between these two groups. This might be because of the unique features of this cytotoxin compared with other toxins of the RTX group (21). Frey and

Nicolet (10) have suggested that two types of hemolysins, a strongly hemolytic HlyI and a much weaker hemolytic HlyII, are found among the various *A. pleuropneumoniae* serotypes. Some of these strains, including serotype 5a and 5b reference strains, have been found to contain both HlyI and HlyII. It is therefore tempting to speculate that the gene *appA* from serotype 5, which was cloned and sequenced by Chang et al. (4), represents the gene encoding HlyII. Indeed, the *appA* gene was cloned by using as a DNA probe the *lktA* gene to which it shows rather strong homology (Table 1).

Immunological data from immunoblot experiments, however, show a different picture. HlyI strongly cross-reacts with the 105-kDa proteins (the putative hemolysins) of all 12 *A. pleuropneumoniae* serotype reference strains but hardly at all with the hemolysins from *E. coli* or the leukotoxin from *Pasteurella haemolytica* (11). These results might be due to the fact that the immunoblot technique uses denatured proteins, in which not all epitopes are interacting with the immune sera.

The alpha-hemolysin of E. coli and the other toxins of the RTX family do not have a classical signal sequence for secretion at their N termini. Their export across the cytoplasmic membrane and the bacterial outer membrane depends on two specific membrane-located secretion proteins, HlyB and HlyD (7, 12, 24, 28, 41). In the case of E. coli alpha-hemolysin, information necessary for secreting an HlyA has been identified as the C-terminal 53 amino acids which are not removed during translocation (16). We have recently shown that the HlyIA protein can be secreted from E. coli when the hlyBD genes are expressed in trans (13). From these data we concluded that the HlyIA protein uses a mechanism for export very similar to that of the E. coli HlyA protein. Since the carboxy termini of HlyIA and HlyA reveal high primary-sequence divergence, these results support the conclusions made by Koronakis et al. (17) that the secretion signal is a particular three-dimensional structure of the molecule's C-terminal end rather than a defined primary amino acid sequence.

The 13 glycine-rich repeated domains found on the amino acid sequence of HlyIA are reminiscent to the capability of binding  $Ca^{2+}$  which was found to be necessary for the E. coli alpha-hemolysin to bind to erythrocytes (1, 2, 23). Our <sup>45</sup>Ca<sup>2+</sup>-binding experiments demonstrate that HlyI as well as the inactive HlyIA is able to bind  $Ca^{2+}$  (Fig. 2) and that  $Ca^{2+}$ binding is independent of activation of HlyIA by the gene product hlyC. By analogy with the E. coli alpha-hemolysin, we attribute this Ca<sup>2+</sup> binding to the 13 glycine-rich repeated sequences. The biosynthesis of A. pleuropneumoniae HlyI has been shown to be induced by  $Ca^{2+}$ , whereas its activity remained nearly unchanged even after treatment with the  $Ca^{2+}$  chelator EGTA [ethylene glycol-bis( $\beta$ -aminoethyl ether)-N,N,N',N'-tetraacetic acid] (8). It seems, therefore, that bound Ca<sup>2+</sup> is not removed from HlyI by the chelator EGTA, indicating a relatively strong Ca<sup>2+</sup>-binding capacity by HlyI. In contrast, HlyII loses its activity with EGTA treatment (8, 10). It is therefore tempting to speculate that the difference in the Ca<sup>2+</sup> requirement for hemolytic activity in HlyI and HlyII could be due to the difference in the number of glycine-rich repeated domains which seem to play a crucial role in  $Ca^{2+}$  binding (2).

In summary, our sequencing results have shown that HlyI has the typical features of the RTX toxins. It has a relatively high divergence from the sequence of a related hemolysin from serotype 5. At least two types of hemolysin are produced by the different *A. pleuropneumoniae* serotypes;

one is very similar to the *E. coli* alpha-hemolysin, and one is more like the *Pasteurella haemolytica* leukotoxin.

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