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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^{2+}-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^{2+} 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^{2+} for its activity but which requires Ca^{2+} 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^{2+} 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* alpha-hemolysin 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*$^+$ *supE44* F^- lambda$^-$ *rpsL20* Sm^r (received

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from P. Prentki, University of Geneva, Geneva, Switzerland). *E. coli* XL1-blue *endA hsdR17 supE44* lambda$^-$ *recA1* del(*proAB-lac*) [F' *proAB lacI^{q}Z*delM15 Tn*10* (Tc^r)] (3) was used for cloning with pBluescript plasmid. *E. coli* 5K *thi-1 thr-1 leu-6 supE44 lacY1 tonA21 hsdR$^-$S$^+$* 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 alpha-hemolysin *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$^-$ (Stratagene, La Jolla, Calif.) was used for subcloning. Plasmid pAP13 (30), which is a pBR322 derivative containing an IS*1* 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 β-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 [^{35}S]dATP (Amersham International plc, Amersham, Buckinghamshire, United Kingdom) and T_7 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_3 primer (Stratagene), 5'-ATTAACCCTCACTAAAG-3'; primer G258, corresponding to the left-end sequence of IS*1*, 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).

$^{45}Ca^{2+}$-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^r) and pLG575 (Cm^r). 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-β-D-thiogalactosidase (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 μg of HlyI) were separated on a 10% polyacrylamide gel (20) and transferred by electrotransfer (33) to nitrocellulose filters (BioRad, Richmond, Calif.). Incubation with $^{45}Ca^{2+}$ was performed as described by Boehm et al. (2). The Ca^{2+}-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 IS*1* 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 *Pvu*II-*Eco*RI 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 IS*1*. A 500-bp segment of *hlyIA* into which an IS*1*-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

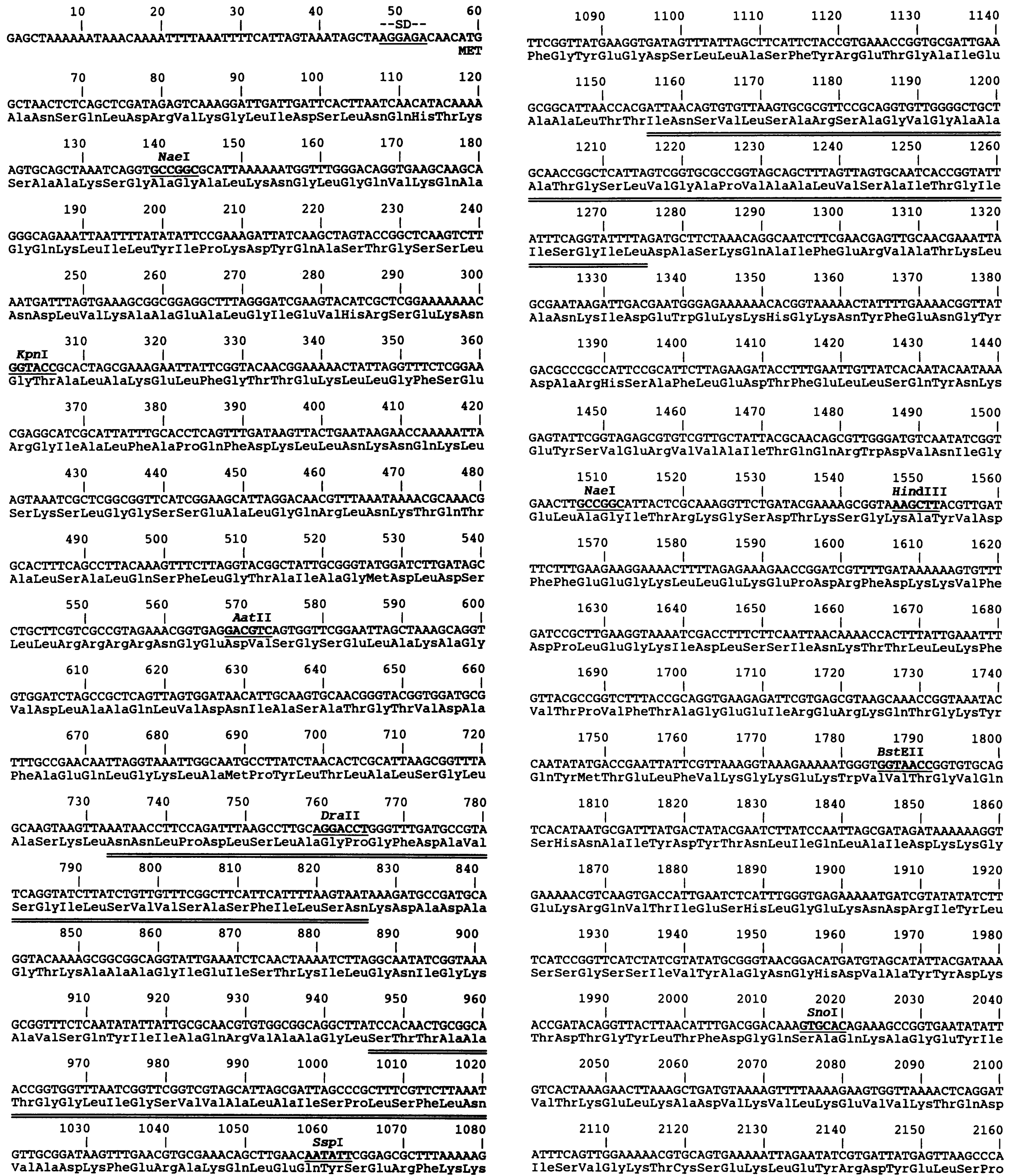

FIG. 1. DNA sequence of *hlyIA* gene and flanking segments from *A. pleuropneumoniae* serotype 1 strain 4074. The sites for some restriction enzymes are shown. The putative ribosome-binding sites are indicated by --SD--. A consensus sequence for a promoter is indicated by <−35> and <−10>. Symbols: ═,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

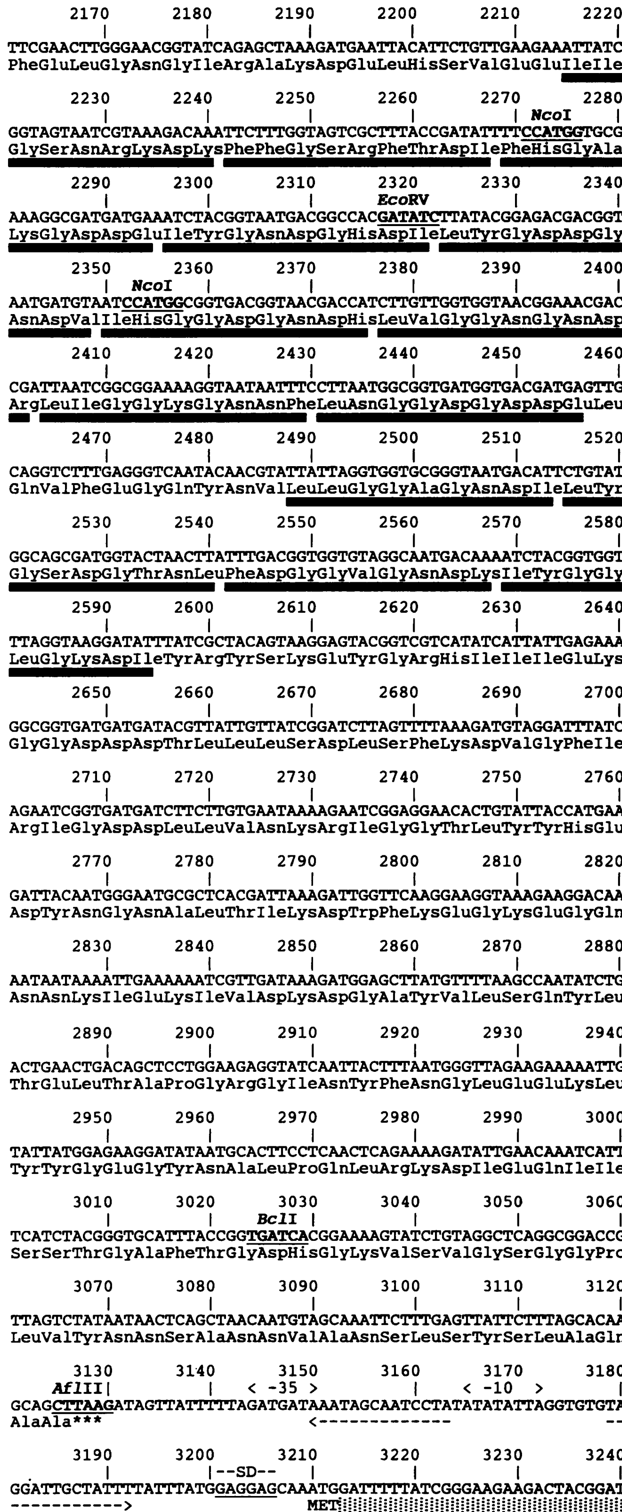

FIG. 1—*Continued.*

TABLE 1. Homologies of *hlyIA* from *A. pleuropneumoniae* serotype 1 to related hemolysins and cytotoxins

Gene[a]	% 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
hlyA	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

[a] 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 consensus-like 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 ΔG 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 HlyIA 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[a]

Repeat	HlyIA	HlyA	AppA	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		

[a] 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^{2+} binding of HlyIA. The capability of HlyI to bind Ca^{2+} was assayed on calcium blots as described by Boehm et al. (2), using $^{45}Ca^{2+}$. Autoradiographs showed that 1,000×-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^{2+}, while the other proteins found in the supernatant did not bind Ca^{2+}. Supernatant of the *A. pleuropneumoniae* serotype 3 reference strain, which does not show hemolytic activity, had no Ca^{2+}-binding protein (results not shown). In order to verify that HlyI was the Ca^{2+}-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^{2+}, 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*

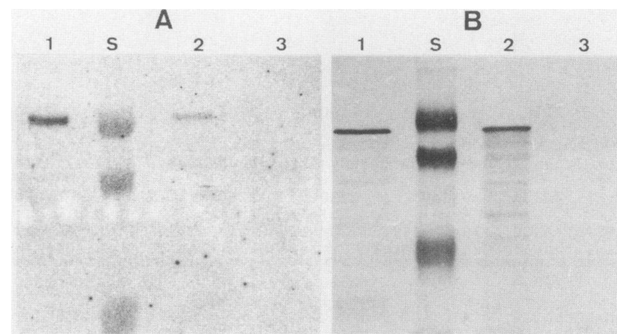

FIG. 2. $^{45}Ca^{2+}$ binding of HlyI and HlyIA. (A) $^{45}Ca^{2+}$ blot; (B) anti-HlyI immunoblot of proteins that were separated on a 10% sodium dodecyl sulfate-polyacrylamide gel and subsequently transferred to nitrocellulose. Lane 1, purified HlyI from *A. pleuropneumoniae* (0.8 μg for $^{45}Ca^{2+}$ blot and 0.2 μg 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 $^{45}Ca^{2+}$ and therefore are most useful on $^{45}Ca^{2+}$ 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. pleuropneumoniae* 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. pleuropneumoniae* 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 $^{45}Ca^{2+}$-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^{2+} 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(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid] (8). It seems, therefore, that bound Ca^{2+} is not removed from HlyI by the chelator EGTA, indicating a relatively strong Ca^{2+}-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^{2+} 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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