hlyIA-Like Sequence Present in a Genetic Locus with Low Homology to appA in Actinobacillus pleuropneumoniae Serotype 10

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ABSTRACT. Using the appA gene encoding Actinobacillus pleuropneumoniae RTX-toxin II (ApxII) as a probe, Southern hybridization demonstrated the presence of appA sequence in all 12 serotypes except serotype 10. Serotype 10 strains had a genetic locus with low homology to appA (tentatively designated Lha). DNA sequence analysis revealed that Lha contained a part of hlyIA gene encoding a 105-kDa hemolysin Apxl. Southern hybridization demonstrated the presence of Lha (hlyIA) sequence not only in serotype 10 but also in serotypes 1, 5a, 5b, 9, and 11, which had strong hemolytic activity with sheep red blood cells and were highly virulent for mice. These results suggest that Apxl may be associated with the virulence phenotype of the organism.—KEY WORDS: Actinobacillus pleuropneumoniae, RTX-toxin gene, virulence.


Actinobacillus pleuropneumoniae is the causative agent of swine pleuropneumonia [29]. Cytotoxins (hemolysins) secreted by this organism into the medium, have been thought to play an important role in virulence [5, 10, 11, 15, 19, 21, 27], and have also been shown to be important components of vaccines for inducing protective immunity [1, 4, 7]. Recently, Kamp et al. [18] identified three different cytotoxic proteins. A. pleuropneumoniae-RTX-toxin I (Apxl [16], also called as ClyI, HlyI; 105 kDa) associated with strong hemolytic activity and cytotoxic activity was produced by serotypes 1, 5a, 5b, 9, 10 and 11, ApxII (ClyII, HlyII, AppA; 103 kDa) associated with weak hemolytic and moderate cytotoxic activities was produced by all serotypes except serotype 10, and ApxIII (ClyIII, Ptx; 120 kDa) associated with strong cytotoxic activity but not with hemolytic activity was produced by serotypes 2, 3, 4, 6 and 8. The functions of these cytotoxins have been described by several workers [6, 22, 24], but their direct role in the pathogenicity of the organism is poorly understood.

The gene encoding Apxl is considered to be hlyIA [9, 14] which was identified in serotype 1 or clyIA [31] in serotype 9. The gene encoding ApxII is considered to be appA [3] which was identified in serotype 5. clyIA [31] in serotype 9 or hlyIA in serotype 2 [13]. ApxI and ApxII proteins belong to the RTX cytotoxin family which is widely distributed among Gram-negative bacterial pathogens in humans and animals [32]. Recently, it has been reported that the gene encoding ApxII is present in the reference strains of all serotypes except serotype 10 and is very similar to each other in these serotypes [13, 17]. On the other hand, the gene encoding Apxl in serotypes 5a, 5b, 9, 10, and 11 has not been characterized.

In this study, we cloned the hlyIA (clyIA)-like sequence from a serotype 10 strain and examined the presence of the sequence among 12 serotypes of A. pleuropneumoniae. In addition, we compared relative virulence of the reference strains of 12 serotypes in mice in an attempt to elucidate a possible relationship between the presence of the sequence and virulence of the organism.

MATERIALS AND METHODS

Bacterial strains and media: The reference strains representing serotypes 1 to 12 of A. pleuropneumoniae were obtained as follows: Strains S1421 (serotype 3), femo (serotype 6), WF83 (serotype 7), CCM3803 (serotype 8), CVI 13261 (serotype 9), and 13039 (serotype 10) were provided by Dr. K. Kume (Research Center for Veterinary Science of The Kitasato Institute, Chiba, Japan); strains 4074 (serotype 1), M62 (serotype 4), K17 (serotype 5a), 56153 (serotype 11) and 1096 (serotype 12) by Dr. K. Yamamoto (National Institute of Animal Health, Ibaraki, Japan); Strain CCM5870 (serotype 2) by Czechoslovak Collection of Microorganisms (Brno, Czechoslovakia) and strain L20 (serotype 5b) by Dr. T. Fukuyasu (School of Veterinary Medicine, Azabu University, Kanagawa, Japan). Strain Ng-2 of serotype 5a was isolated from the lungs of a diseased pig at our institute. Field isolates CV122009 and 744 of serotype 10 [20] were provided by Dr. K. R. Mittal (University of Montreal, Saint-Hyacinthe, Quebec, Canada). Escherichia coli strain XL1-Blue (endA1, hsdR17, supE44, thi-1, recA1, gyrA96, relA1, del(protAB-lac) [F', proAB, lacFZdelI5, Tn10(Tc)]) was used in transformation experiments [2]. Plasmid pBluescript II KS+ (Stratagene, La Jolla, CA) was used for the gene cloning. A. pleuropneumoniae strains were grown in heart infusion broth (Infusion broth, BBL, Cockeysville, MD) supplemented with 0.3% yeast extract (Dried yeast extract-S, Nippon Seiyaku Co., Ltd., Tokyo, Japan) and 0.0025% beta-nicotinamide-adenine dinucleotide (Oriental Yeast Co., Ltd., Tokyo, Japan). E. coli was grown in Luria-Bertani broth or agar [23], and the medium for the transformants was supplemented with ampicillin sodium (50 μg/ml).

Gene cloning: The appCA genes of strain Ng-2 (sero-
type 5a) were cloned into pBluescript II KS+ vector (Stratagene, La Jolla, CA) by the chromosome walking method [23] with synthetic oligonucleotides (5'-ACCGGTAATGATATTATCTCGGAGGTAAA-3') corresponding to a nucleotide position of 2841–2870 in the \textit{appA} gene [3]. The cloned fragment containing \textit{appCA} from strain Ng-2 is shown in Fig. 1A. Analysis of the nucleotide sequence of the cloned \textit{appCA} genes revealed identity to the sequence of the hemolysin gene characterized by Chang et al. [3]. A 2.0-kb \textit{EcoRV-KpnI} fragment, which was hybridized to the \textit{appA} gene under a low stringent condition, was cloned into pBluescript II as follows: The genomic DNA of \textit{A. pleuropneumoniae} strain 13039 (serotype 10) was completely digested with \textit{EcoRV} and \textit{KpnI}. The resulting fragments were separated by electrophoresis on 0.7% agarose gels. Desired fragments were eluted from gel slices and ligated to the \textit{EcoRV} and \textit{KpnI}-digested and dephosphorylated vector. The transformants harboring the plasmid containing the desired fragment were obtained by the colony hybridization method [23] using \textit{appA} as a probe under a low stringent condition.

\textit{Southern blot analysis}: A 1.9-kb \textit{PstI-PstI} fragment (Fig. 1A) containing a central part of the \textit{appA} gene was used for detecting sequence homologous to the \textit{appA} gene. A 1.5-kb \textit{XbaI-XbaI} fragment (Fig. 1A) containing 3' half of \textit{appC} and 5' half of \textit{appA}, and a 2.1-kb \textit{XbaI-XbaI} fragment (Fig. 1A) containing 3' half of the \textit{appA} gene and 0.5 kb downstream from the \textit{appA} gene were used to detect sequence homologous to 5' and 3' half of the \textit{appA} gene, respectively. A 2.0-kb \textit{KpnI-EcoRV} fragment containing the genetic locus with low homology to \textit{appA} was used to detect sequence homologous to this locus. The relevant fragments were purified by agarose gel electrophoresis and labeled with [alpha-P\textsuperscript{32}]-dATP (Amer sham Corp., Arlington Heights, IL) by a random prime labeling kit (Boehringer GmbH., Mannheim, Germany). \textit{A. pleuropneumoniae} genomic DNA, which had been prepared by the method of Smith et al. [30], was digested with \textit{PstI} or \textit{XbaI}, electrophoresed through a 0.7% agarose gel, and transferred to a nitrocellulose filter. For a high stringent condition, blots were hybridized in 50% formamide, 5 × SSC (1 × SSC is 0.15 M NaCl, 0.015 M Na citrate, pH 7.0), 5 × Denhardt's solution (1 × Denhardt's solution is 0.02% Ficoll, 0.02% bovine serum albumin, 0.02% polyvinylpyrrolidone), 0.1% SDS, and 100 μg/ml sheared salmon sperm DNA for 14 hr at 42°C. Then the filters were washed twice with 2 × SSC-0.1% SDS for
15 min at room temperature, and washed finally with 0.1× SSC-0.1% SDS for 30 min at 50°C. For a low stringent condition, blots were hybridized in the same solution for 14 hr at room temperature. Then the filters were washed twice with 2× SSC-0.1% SDS for 15 min at room temperature. After being washed, the filters were subjected to autoradiography.

**DNA sequencing:** The sequences of both DNA strands were determined by the dideoxy chain-termination method [28], using a non-radio isotope DNA sequencing kit Sequencing high (Toyobo Co., Ltd., Osaka, Japan).

**Hemolytic activity:** The reference strains of *A. pleuropneumoniae* grown overnight in broth medium were diluted 1:20 with fresh broth medium and incubated with shaking at 37°C for 2 hr. The incubated cultures were centrifuged for 10 min at 10,000 × g, and the supernatant (0.25 ml) was added to an equal volume of 1% sheep red blood cells (SRBC) in phosphate-buffered saline pH 7.2 (PBS). After incubation for 2 hr at 37°C, the mixture was centrifuged for 10 min at 2,000 × g, and the optical density (OD) of the supernatant was determined at a wavelength of 540 nm. An OD value of 0.1 or greater was regarded as positive.

**Determination of fifty percent lethal dose (LD<sub>50</sub>) of *A. pleuropneumoniae* in mice:** Three-week-old outbred female mice of ddY (Japan SL Co., Ltd., Hamamatsu, Japan) were used. The overnight cultures of the reference strains of the 12 serotypes were diluted 1:20 with fresh broth media and incubated with shaking at 37°C for 3 hr. Serial decimal dilutions of the cultures were made in PBS. Colony-forming units (CFU) of the organisms in each dilution were quantitated by culturing them on agar plates. The inoculum for mice consisted of one volume of each dilution and 9 volumes of 10% mucin (Sigma, ST Louis, MO). Ten mice were inoculated intraperitoneally with 0.5 ml of each dilution. Control mice received a mixture of PBS and 10% mucin. Inoculated mice were observed for mortality for 7 days. Mice that died were necropsied and examined for the presence of *A. pleuropneumoniae*. The LD<sub>50</sub> was calculated for each serotype by the method of Reed and Muench [25].

**RESULTS**

**Distribution of the appA gene in the reference strains of serotypes 1 to 12:** To verify the distribution of ApxI, we examined the presence of the *appA* sequence in 12 serotypes of *A. pleuropneumoniae*. The reference strains of all serotypes except serotype 10 were shown to have a DNA fragment homologous to the *appA* gene by Southern hybridization using a 1.9-kb *PstI*-PstI fragment as a probe (Figs. 1A, B and C). This hybridization also showed that all the serotype strains except serotype 10 uniformly possessed both 1.9-kb *PstI*-PstI fragment (Fig. 1B) and 1.5-kb *XbaI*-XbaI fragment (Fig. 1A) homologous to *appA*. This indicated that the two *PstI* sites and the two *XbaI* sites located in the *appA* gene were conserved in these serotype strains.

The presence of the entire gene of *appA* was examined in three strains of serotype 10 by Southern hybridization using the probes containing all the open reading frame of the *appA* gene and its flanking region. None of these strains had a DNA fragment homologous to any of these probes (Figs. 2A, B and C). A weak hybridization was detected in the reference strain of serotype 10 only when hybridization was performed under a low stringent condition (Fig. 2D). Both 2.1- and 1.5-kb *XbaI*-XbaI fragments were detected in two strains of serotypes 5 and 2, while an
approximately 4.7-kb fragment was detected in the serotype 10 strain. This indicated that the serotype 10 strain had a unique genetic locus, which was tentatively designated Lha, with low homology to appA.

Cloning and DNA sequence analysis of the Lha locus: A 2.0-kb KpnI-EcoRV fragment containing the Lha locus was cloned into a plasmid vector. Using the cloned DNA fragment as a probe, Southern hybridization showed the presence of the Lha sequence in three strains of serotype 10 (Fig. 3B). This hybridization also showed that the reference strain of serotype 2 did not have the Lha sequence, but unexpectedly, the serotype 1 strain had this sequence. The appA sequence was detected in two reference strains of serotypes 1 and 2, but not in three strains of serotype 10 (Fig. 3A). In the serotype 1 strain, which hybridized to both probes, the DNA fragment hybridized to Lha was larger than that hybridized to appA.

As the Lha sequence was detected not only in serotype 10 but also in serotype 1, this sequence might be related to the hlyIA gene encoding Apxl protein. The DNA sequence analysis showed that the Lha locus was almost identical to a part of the hlyIA gene of serotype 1 from the KpnI site at coordinate 305 bp to the EcoRV site at coordinate 2,319 bp for the sequence [9]. The predicted amino acid sequence of the locus differed from HlyIA at seven positions: At amino acid position 374 arginine in HlyIA was substituted for alanine in the Lha locus, at position 491 serine for alanine, at position 493 threonine for alanine, at position 512 glutamic acid for aspartic acid, at position 542 valine for isoleucine, at position 562 glutamine for glutamic acid, and at position 581 glutamine for glutamic acid.

Distribution of the Lha (hlyIA) sequence among the reference strains of 12 serotypes of A. pleuropneumoniae was examined by Southern hybridization under a stringent condition. An EcoRI-EcoRI 8.5-kb fragment homologous to the Lha locus was detected in the reference strains of serotypes 1, 5a, 5b, 9, 10, and 11 (Fig. 4). The presence of the Lha (hlyIA) and appA sequences in the reference strains of serotypes 1 to 12 of A. pleuropneumoniae is shown in Table 1. Serotypes 1, 5a, 5b, 9, and 11 had both appA and Lha (hlyIA) sequences. Serotypes 2, 3, 4, 6, 7, 8, and 12 had the appA sequence only and the serotype 10 strains had the Lha (hlyIA) sequence only.

Phenotypes relating to the Lha (hlyIA) sequence: To examine the relationship between the presence of the appA or Lha (hlyIA) sequence and virulence phenotypes of this organism, we investigated the hemolytic activity with SRBC and virulence for mice in the reference strains of the 12 serotypes. Hemolytic activities of the culture supernatants of serotypes 1, 5a, 5b, 9, 10, and 11 were much stronger than those of serotypes 2, 3, 4, 6, 7, 8, and 12 (Table 1). Log LD₉₀ in mice varied among strains as shown in Table 2. Serotypes 1, 5a, 5b, 9, 10, and 11 showed relatively low LD₉₀ ranging from 4.8 to 5.9 log CFU, while LD₉₀ of the remaining serotypes ranged from 7.9 to 9.0 log CFU. These findings indicated that the virulence of serotypes 1, 5a, 5b, 9, 10 and 11 was higher than that of the other serotypes in mice. These results are summarized in Table 1.

DISCUSSION

Our Southern hybridization demonstrated that the reference strains of all 12 serotypes except serotype 10 of A. pleuropneumoniae had the appA [3] sequence on their chromosome. This hybridization also showed that two PstI sites and two XbaI sites were conserved in these 11 serotypes (Figs. 1A, B and C), indicating a high similarity of the appA gene in these serotypes. The reference strain
of serotype 10 (strain 13039) has been reported to lack the clyIA (appA) gene and not to produce ApxII protein [17]. Our data agree with these results. To clarify whether the lack of the appA gene was observed only in the reference strain of serotype 10 or was commonly detected in this serotype strains, two field isolates of serotype 10 were examined for the presence of the appA sequence. Southern hybridization demonstrated that all the serotype 10 strains tested lacked the entire appA gene, suggesting that the appA gene may be commonly absent in serotype 10 strains.

When hybridization was performed under a low stringent condition, the fragment homologous to appA was barely detected in the serotype 10 strains, and the number and size of the DNA fragment apparently differed from those of other serotype strains. This result indicates that the serotype 10 strains have a unique genetic determinant sharing low homology with appA. We cloned the 2.0-kb KpnI-EcoRI fragment containing the Lha locus hybridized to the appA gene at a low stringent condition. The Southern hybridization using this DNA fragment as a probe demonstrated that the Lha (hlyIA) sequence was present not only in serotype 10, but unexpectedly, also in the serotypes 1, 5a, 5b, 9 and 11. This hybridization also indicated that the serotype 1 strain possessed both Lha and appA sequences that were located in a different DNA fragment. Our observation suggests that the Lha locus may be related to the hlyIA [9, 14] gene encoding ApxI protein for the following reasons. 1) hlyIA was shown to share 50.7% homology to appA [9], identical to the Lha locus sharing low homology to appA. 2) The distribution of the Lha locus was the same as that of hlyIA [8] among the 12 serotypes of A. pleuropneumoniae. DNA sequence analysis of the Lha locus revealed that this locus was almost identical to a part of the hlyIA gene from a KpnI site at coordinate 305 bp to an EcoRV site at coordinate 2319 bp for the DNA sequence [9]. Although the predicted amino acid sequences of Lha and hlyIA were different at seven positions in 671 amino acid residues, the structure of the protein in this region was supposed to be almost similar by a computer analysis. The present result showed that we cloned a part of the gene encoding ApxI from serotype 10 strain. The molecular cloning and the determination of the entire sequence of the gene in serotype 10 are now in progress.

The Lha (hlyIA) sequence was demonstrated not only in serotypes 1 and 10 but also in serotypes 5a, 5b, 9 and 11. Our hybridization study using appA as a probe revealed the presence of the appA gene in serotypes 1, 2, 3, 4, 5a, 5b, 6, 7, 8, 9, 11, and 12. Therefore, the reference strains of serotypes 1, 5a, 5b, 9, and 11 were shown to have both Lha and appA sequences (Table 1). The distributions of the appA gene and Lha (hlyIA) sequences among the reference strains of the 12 serotypes were the same as those of ApxII and ApxI proteins, respectively (Table 1). These results confirmed the distribution of ApxI and ApxII among A. pleuropneumoniae serotypes.

### Table 1. Presence of appA and Lha (hlyIA), biological phenotypes, and production of cytotoxins in the reference strains of serotypes 1 to 12 of A. pleuropneumoniae

<table>
<thead>
<tr>
<th>Serotype</th>
<th>appA (hlyIA)</th>
<th>Lha (hlyIA)</th>
<th>Hemolytic activity</th>
<th>Virulence for mice</th>
<th>ApxI&lt;sup&gt;a&lt;/sup&gt;</th>
<th>ApxII&lt;sup&gt;a&lt;/sup&gt;</th>
<th>ApxIII&lt;sup&gt;a&lt;/sup&gt;</th>
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<sup>a</sup> Kamp et al. [18].

<sup>b</sup> Presence.

### Table 2. Fifty percent lethal dose (LD<sub>50</sub>) of the reference strains of A. pleuropneumoniae serotypes 1 to 12 in mice

<table>
<thead>
<tr>
<th>Serotype</th>
<th>LD&lt;sub&gt;50&lt;/sub&gt;&lt;sup&gt;a&lt;/sup&gt;</th>
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<sup>a</sup> Log CFU.
We compared the presence of the Lha (hlyA) and appA sequences with hemolytic activity and virulence for mice among the reference strains of the 12 serotypes. All the serotype strains with the Lha (hlyA) sequence showed strong hemolytic activity with SRBC and were highly virulent for mice. In contrast, none of the remaining serotypes without the Lha (hlyA) sequence showed hemolytic activity and they were less virulent for mice. Although serotypes 2, 3, 4, 6, 7, 8, and 12 have been reported to have a weak hemolytic activity [12], we could not detect hemolytic activity in these serotypes as has been reported by different groups of workers [19]. This discrepancy may be due to different buffers used in the assay. We used PBS without adding Ca²⁺ which has been reported to be required for hemolytic activity in these serotypes [12]. For this reason, the presence of hemolytic activity in these serotypes may not be denied. Our results are consistent with those of Komal and Mittal [20] who reported that strains of serotypes 1, 5, 9, 10, and 11 were highly virulent in CD1 mice by intraperitoneal and intranasal inoculations. Their results along with ours strongly suggest that the Lha (hlyA) sequence associated with strong hemolytic activity may be responsible for the virulence of this organism in mice.

A few studies have focused on the pathogenicity of A. pleuropneumoniae serotypes in pigs. Rogers et al. [26] reported that field isolates of serotype 1 were more virulent for pigs than serotypes 2, 3, and 7. This may be explained by the present observation that the Lha (hlyA) sequence exists in serotype 1 strains but not in strains of serotypes 2, 3, and 7. In our preliminary test, the reference strain of serotype 10, which lacks the appA but has the Lha (hlyA) sequence, showed high virulence in pigs following intratracheal inoculation with 10⁶ CFU per pig (Nagai, S., unpublished data). This leads us to speculate that the Lha (hlyA) may be associated with virulence in pigs as observed in mice. Further studies on the biological and biochemical functions of each gene product are obviously needed to elucidate the role played by these genes in pathogenicity.

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