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Molecular Characterization of an RTX Toxin Determinant from *Actinobacillus suis*

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RTX cytolysins are a family of calcium-dependent, pore-forming, secreted toxins found in a variety of gram-negative bacteria. The prototypical member of this family is the α-hemolysin of *Escherichia coli*. The RTX genetic determinants from seven members of the family *Pasteurellaceae*, *Pasteurella haemolytica*, *Actinobacillus actinomycetemcomitans*, and *A. pleuropneumoniae* serotypes 1, 5, 7, and 9 were previously cloned and sequenced. Using the leukotoxin determinant from *P. haemolytica* serotype A1 as a probe, we detected the presence of RTX-type determinants in *Actinobacillus suis*, *A. equuli*, and *A. lignieresii* of the family *Pasteurellaceae*. All three species elaborate proteins of approximately 104 to 110 kDa that are recognized by polyclonal antisera against the 104-kDa hemolysin of *A. pleuropneumoniae* serotype 1. An RTX determinant of *A. suis* isolate 3714 was cloned and sequenced and was found to be almost identical to the RTX determinant of *A. pleuropneumoniae* serotypes 5 and 9. In addition, the determinant is not composed of four contiguous genes, as had been reported for most other RTX determinants; instead, the genes encoding the two proteins responsible for secretion of the toxin are at a locus distinct from that containing the toxin structural and activation genes.

Species of the genus *Actinobacillus* are gram-negative capnophilic coccobacilli belonging to the family *Pasteurellaceae* (33). They are commensals and opportunistic pathogens of swine, cattle, and horses. *Actinobacillus lignieresii* is the type species of the genus and was first described by Lignieres and Spitz in 1903 (41). It is the etiological agent of actinobacillosis (wooden tongue) of cattle. *Actinobacillus suis* was first described by Terpstra and Akkermans in 1955 and named *A. suis* by van Dorssen and Jaartsveld (30). *A. suis* and *Actinobacillus equuli* are closely related species, distinguished by biochemical differences including carbohydrate fermentation and esculin hydrolysis and the production of β-hemolysis by *A. suis* on sheep blood agar (33).

Disease in swine infected with *A. suis* usually occurs in young animals (less than 6 months of age) and frequently manifests as acute fatal septicemia (30). Disease in older animals occurs less frequently. Swine herds infected with *A. suis* are not uncommon, but the ability of this organism to cause disease appears to be limited (30). Septicemia caused by hemolytic actinobacilli in horses has also been reported (4, 6, 22, 25), but the strains involved have usually been described as hemolytic variants of *A. equuli*.

Recently, members of the *Pasteurellaceae*, including *Pasteurella haemolytica*, *Actinobacillus pleuropneumoniae*, and *A. actinomycetemcomitans*, have been shown to possess genetic determinants encoding pore-forming protein toxins belonging to the RTX (repeats in the structural toxin) cytolysin family (7, 11, 14, 24, 26). The products of four separate genes are required for the elaboration of these cytolysins: the toxin structural gene (*A*), a gene encoding a protein required for activation of the toxin (*C*), and two genes coding for a toxin secretion function (*B* and *D*) (44). The cytolysins have a unique region of repeating glycine-rich domains thought to be involved in binding calcium, which is required for target cell-binding or cytolytic activity (44). The RTX family also includes the α-hemolysin of *Escherichia coli* (12, 18, 43), one of the hemolysins of *Proteus vulgaris* (23), the cyclolysin of *Bordetella pertussis* (15), and a 180-kDa protein from *Neisseria meningitidis* (28), among others. The cytolysins are thought to contribute to the virulence of these bacteria (14, 42). We show that three additional members of the family *Pasteurellaceae*, *A. equuli*, *A. suis*, and the type species of *Actinobacillus*, *A. lignieresii*, possess RTX cytolysin genes. An RTX hemolysin determinant of *A. suis* 3714 is characterized by using Southern blot and Western immunoblot analyses and DNA cloning and sequencing.

MATERIALS AND METHODS

Bacteria, vectors, and culture conditions. The bacterial strains used in this study are described in Table 1. The vectors used for cloning (pBR322) and sequencing (M13 mp18 and mp19) were previously described (26). The recombinant plasmid pSF4000, containing the *E. coli* hemolysin determinant, was obtained from Rod Welch, Madison, Wis. (12).

Actinobacillus strains were maintained on sheep blood agar and grown in Todd-Hewitt broth (Difco Laboratories, Detroit, Mich.) supplemented with 10 mM $CaCl_2$ (THB+ Ca). *E. coli* strains were maintained on LT plates and grown in LT medium or LT supplemented with antibiotics as previously described (26). *E. coli* TG-1, used as a host for the propagation of the M13 phage, was grown in Davis minimal medium as described previously (27).

Rabbit antiserum prepared against the gel-purified 104-kDa hemolysin of *A. pleuropneumoniae* serotype 1 was the generous gift of Soren Rosendal, Guelph, Ontario, Canada (11).

Enzymes and chemicals. Restriction endonucleases and DNA-modifying enzymes were purchased from Bethesda Research Laboratories (Burlington, Ontario, Canada), or Pharmacia Chemicals, Inc., and were used as described by the suppliers. Radioisotopes were purchased from ICN Biomedical (Montreal, Quebec, Canada). Goat anti-rabbit immunoglobulin G-alkaline phosphatase conjugate and color

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TABLE 1. Identification and source of strains

Strain	Source or reference
A. equuli clinical isolates (from horses)	
1436	S. Rosendal
1964	S. Rosendal
5012	S. Rosendal
A. lignieresii isolates (from cattle)	
K27	S. Rosendal
4191	NCTC[a]
A. suis isolates (from swine[b])	
1 (horse)	J. Prescott
3 (horse)	J. Prescott
7	J. Prescott
K28	S. Rosendal
777	S. Rosendal
2586	S. Rosendal
3714	S. Rosendal
15558 (reference strain)	ATCC[c]
P. haemolytica A1	26
E. coli	
HB101	26
TG-1	27

[a] NTCC, National Collection of Type Cultures.
[b] Unless otherwise indicated.
[c] ATCC, American Type Culture Collection.

development reagents were purchased from Bio-Rad Laboratories (Mississauga, Ontario, Canada).

Crude hemolytic assays. The hemolytic activity of culture supernatants was measured by a crude liquid assay. Briefly, erythrocytes (bovine, canine, equine, ovine, or porcine) were harvested from fresh blood by centrifugation at 1,500 × *g* in a Beckman clinical centrifuge. The erythrocytes were washed three times in an equal volume of 0.85% saline–10 mM $CaCl_2$. The washed erythrocytes were diluted to 1% in 0.85% saline–10 mM $CaCl_2$ for use in the assay. Bacteria were grown overnight in THB+Ca at 37°C, subcultured (diluted 1/100) in the same medium, and then grown for 4 h at 37°C. After 4 h, the cells were removed from the supernatant by centrifugation at 12,100 × *g* and then filtered through a low-protein-binding Millex-HV 0.45-μm-pore-size filter (Millipore, Bedford, Mass.). The filtered supernatant was mixed in a 1:4 ratio with the 1% erythrocyte suspension, and the mixture was incubated for 60 min at 37°C. The mixtures were then centrifuged at 12,000 × *g* to pellet the erythrocytes. The release of hemoglobin was measured by determining the optical density of the supernatant at 540 nm with a Philips Pye Unicam PU86000 spectrophotometer. Hemolytic activity was calculated as the percent hemolysis compared with a 100% lysed positive control containing a 1:4 ratio of THB+Ca to 1% erythrocyte suspension plus 0.001% Triton X-100. A 1:4 ratio of THB+Ca to 1% erythrocyte suspension was used as a negative control.

Antibody neutralization studies were performed by preincubating the culture supernatant with serial dilutions of anti-*A. pleuropneumoniae* hemolysin antibody for 30 min at room temperature and then performing hemolytic assays as described above.

Preparation of supernatant proteins and Western blot analysis. For Western immunoblot analysis, the bacterial strains were grown overnight in THB+Ca, subcultured (diluted 1/100) in the same medium, and grown for 4 h at 37°C on a rotary shaker at 150 rpm. The cells were removed from the supernatant by centrifugation and filtration as described above. Supernatant proteins were precipitated either by adding trichloroacetic acid (6% final concentration) or ammonium sulfate (60% saturation). The precipitate was collected by centrifugation, washed in cold acetone, and resuspended in 2× sodium dodecyl sulfate (SDS) sample buffer (0.125 M Tris-HCl [pH 6.8], 4% SDS, 20% glycerol, 10% 2-mercaptoethanol, 0.05% bromophenol blue). These samples were analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) and Western blotting as previously described (26). Rabbit antiserum against the purified 104-kDa hemolysin of *A. pleuropneumoniae* serotype 1 was used to detect homologous proteins in the Western blot assays.

Agarose gel electrophoresis and Southern blot analysis of *Actinobacillus* DNA. *Actinobacillus* chromosomal DNA was isolated by SDS lysis of an overnight culture followed by phenol extraction and ethanol precipitation as previously described (26). Restriction endonuclease digests of genomic DNA and recombinant plasmids were analyzed by electrophoresis in 0.7% agarose gels. For Southern blot analysis, *Actinobacillus* genomic DNA was digested with restriction endonucleases, separated on a 0.7% agarose gel, and electrophoretically transferred to nitrocellulose paper. Probes were made by enzymatic digestion of pLKT52 (which contains the *P. haemolytica* leukotoxin genes) (40) and recovery of the appropriate fragments after separation in low-melting-point agarose gels. The probes were labeled with [^{32}P]dATP by nick translation and hybridized as previously described (26), except that 25% formamide was used.

DNA cloning and sequencing. *Actinobacillus* chromosomal DNA was digested with the restriction endonuclease *Cla*I or *Hin*dIII, and fragments of 5 to 10 kbp were selected after centrifugation through a 10 to 40% linear sucrose gradient. These fragments were ligated into the unique *Cla*I or *Hin*dIII site of the plasmid vector pBR322, and the recombinant plasmids were transformed into *E. coli* HB101. *E. coli* clones carrying recombinant plasmids (identified by loss of tetracycline resistance) were screened for DNA fragments with homology to the *lkt* determinant of *P. haemolytica* by using a colony blot assay. Briefly, the recombinant clones were grown on nitrocellulose filters overlaid on LT-ampicillin plates and then lysed with 0.5 M NaOH; the DNA was then precipitated with ethanol. The nitrocellulose filters were probed with [^{32}P]ATP-labeled *lkt* determinant. The recombinant plasmids of positively hybridizing clones were prepared by CsCl-ethidium bromide density gradient centrifugation and stored at −20°C.

After digestion of inserts from the recombinant plasmids with appropriate restriction enzymes, the DNA fragments were purified after low-melting-point agarose gel electrophoresis and ligated into M13 mp18 and mp19 vectors digested with appropriate restriction enzymes. The phage DNA was amplified in *E. coli* TG-1, purified by phenol-chloroform extraction, and sequenced by the dideoxy chain termination method as previously described (27). Overlapping deletions of insert DNA were created as previously described (27) to facilitate sequencing of large fragments. In most cases, each nucleotide was sequenced three times from different deletion endpoints. Analysis and comparisons of DNA sequence data were performed by using the Pustell Sequence Analysis programs (International Biotechnologies Inc., Toronto, Ontario, Canada) as described previously (27).

Nucleotide sequence accession number. The DNA sequence of *ashC* and *ashA* has been deposited in the GenBank library under accession number M90440.

RESULTS

Hemolytic assays. Despite the genetic and immunological similarities among the RTX cytolysins, they vary in their target cell specificities. For example, the *A. actinomycetemcomitans* leukotoxin lyses only leukocytes of humans and Old World monkeys (24), whereas the hemolysin of *A. pleuropneumoniae* can lyse both erythrocytes and leukocytes from a variety of animal species (3).

With a crude liquid assay, 4-h culture supernatants from various strains of *A. suis* were found to cause almost complete lysis of bovine, porcine, equine, and ovine erythrocytes. A similarly wide target cell range has been described for the *E. coli* and *A. pleuropneumoniae* hemolysins (3, 13, 36). Rabbit antiserum against the 104-kDa hemolysin of *A. pleuropneumoniae* serotype 1 neutralized this cytolytic activity (results not shown).

Southern blot hybridization. With the entire cloned *lkt* determinant from *P. haemolytica* A1 as a probe, chromosomal DNA from various clinical strains of *A. equuli*, *A. lignieresii*, and *A. suis* were screened for the presence of RTX determinants. Most of the strains tested showed positive hybridization signals, although the pattern of restriction endonuclease fragments was different among species and sometimes among different strains of the same species (31) (Fig. 1). Most of our clinical isolates of *A. suis*, including the one chosen for further characterization, had restriction endonuclease patterns similar to that of the American Type Culture Collection reference strain, 15558. Two of the *A. suis* isolates were equine (Fig. 1a, lanes 2 and 3) rather than porcine (Fig. 1a, lanes 4 through 9) strains. Although they were phenotypically similar to the other strains, their chromosomal DNAs showed different restriction endonuclease patterns (restriction fragment length polymorphisms) and they produced hemolysins of a larger molecular weight. These strains may represent hemolytic variants of *A. equuli* (4, 6, 22) and were not further characterized.

Western immunoblot analysis. The production of RTX proteins by various strains of *A. suis*, *A. equuli*, and *A. lignieresii* was examined by using rabbit polyclonal antiserum prepared against the 104-kDa protein from *A. pleuropneumoniae* serotype 1. In *A. suis* cultures, a protein of ca. 105 kDa was detected in the supernatant approximately 4 h after subculture (representing the log phase) (Fig. 2). In contrast, no proteins reacting with the *A. pleuropneumoniae* antiserum were detected in the *A. equuli* or *A. lignieresii* log-phase culture supernatants or in lysed whole cells after a similar incubation period. The leukotoxin of *P. haemolytica* and the *E. coli* α-hemolysin were also detected by this antiserum (Fig. 2). The American Type Culture Collection reference strain of *A. suis* 15558, and most of our clinical isolates from swine produced a hemolysin of ca. 105 kDa, whereas two equine isolates (strains 1 and 3) produced a larger protein of ca. 112 kDa (data not shown).

Cloning and sequencing of the *ashC* and *ashA* genes of *A. suis*. With the *lkt* determinant of *P. haemolytica* as a probe, a positively hybridizing 7.5-kb *Cla*I chromosomal DNA fragment from *A. suis* 3714 was cloned into pBR322 and maintained in *E. coli* HB101. Initial sequence analysis indicated that an *lktC* analog gene and approximately 80% of an *lktA* analog gene were present in this clone, p3714G (Fig. 3). These genes were designated *ashC* and *ashA*, respectively (for *A. suis* hemolysin). By using a 0.8-kb *Hin*dIII-*Cla*I fragment from the 3′ end of the insert of p3714G, a 2.5-kb *Hin*dIII overlapping chromosomal DNA fragment was cloned into pBR322, forming pHC3-1. This clone contained

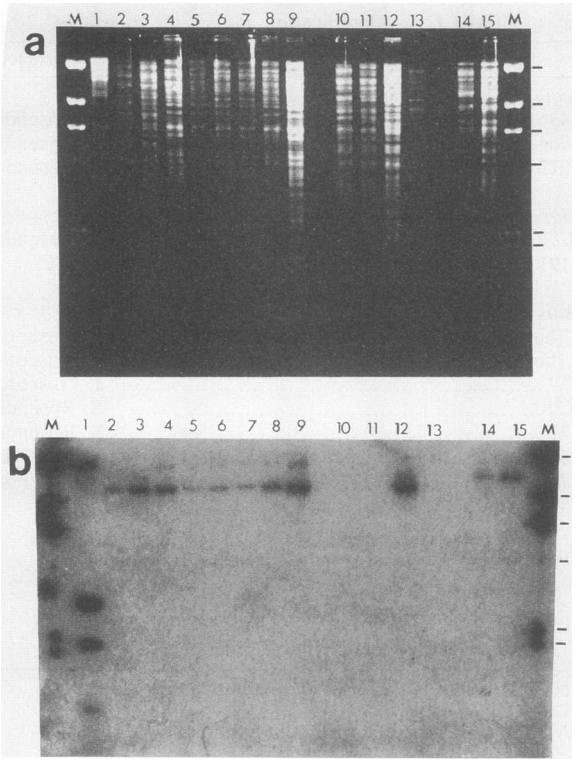

FIG. 1. Southern blot analysis of chromosomal DNA from various members of the family *Pasteurellaceae*. Chromosomal DNA was digested with *Eco*RV, separated on a 0.7% agarose gel, and blotted onto nitrocellulose. The blot was probed with the *P. haemolytica lkt* determinant at low stringency. (a) Agarose (0.7%) gel stained with ethidium bromide; (b) autoradiogram of a Southern blot. Lanes: M, bacteriophage lambda DNA digested with *Hin*dIII, giving fragments of 23.0, 9.4, 6.7, 4.4, 2.3, and 2.0 kb from the top down; 1, *P. haemolytica* A1; 2 through 9, *A. suis* 1, 3, 7, K28, 777, 2586, 3714, and 15558, respectively; 10 through 13, *A. equuli* 1436, 3700, 1964, and 5012, respectively; 14 and 15, *A. lignieresii* K27 and 4191, respectively.

the remaining 20% of the *ashA* gene and the beginning of a putative *ashB* gene (Fig. 3). The inserts of p3714G and pHC3-1 were subcloned and ligated into pBR322 to form pAcsAC-61, which contains *ashC*, *ashA*, and an incomplete copy of *ashB*. However, this clone does not produce any hemolytic activity; the reason for this lack of hemolytic activity is being investigated.

The coding regions of the inserts of p3714G and pHC3-1 were sequenced. Comparison of the *ashC* and *ashA* DNA sequences with those of *A. pleuropneumoniae appC/A* (7) and *clyIIC/A* (38) showed that the sequences were almost identical. The *ashC* and *clyIIC* genes are 100% homologous. The minor differences between *ashC* and *appC* are the same as those described by Smits et al. (38) for *clyIIC* and *appC*. For example, the discrepancy that occurs at positions 127 through 140 of *appC/ashC* is due to a frameshift in this region. Note that the *ashC/clyIIC* version gives a deduced amino acid sequence that more closely matches the RTX consensus (Fig. 4).

The nucleotide sequence data showed that *appA* and *clyIIA* are 100% identical, whereas the *ashA* sequence differs from them at three positions. These alterations in the DNA sequence are reflected in changes in the deduced amino acid sequence (Fig. 4). This degree of homology is the highest yet

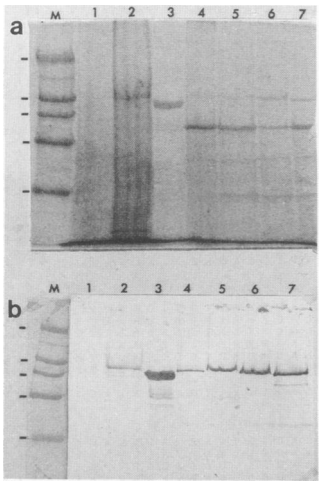

FIG. 2. Western blot analysis of precipitated supernatant proteins from various *A. suis* strains. Supernatant proteins were acid precipitated from log-phase culture supernatants and washed with acetone (see Materials and Methods). Proteins were separated on SDS-PAGE gels (7.5% polyacrylamide) and blotted onto nitrocellulose. The blot was probed with polyclonal rabbit antiserum prepared against the 104-kDa hemolysin of *A. pleuropneumoniae* serotype 1. (a) SDS-PAGE gel stained with Coomassie blue; (b) Western blot. Lanes: M, molecular weight markers of 200, 116, 97, 68, and 43 kDa from the top down stained with amido black; 1, *E. coli* HB101; 2, *E. coli* HB101 carrying pSF4000; 3, *P. haemolytica* A1; 4 through 7, *A. suis* K28, 777, 3714, and 15558, respectively.

reported for RTX determinants from different species. To dispel any doubts regarding the possible misidentification of *A. suis* 3714 in light of the high degree of homology detected, the strain was subjected to a number of biochemical tests and was positively reidentified as a strain of *A. suis*.

Detection of the *ashB* and *ashD* genes. Next to the stop codon at the 3′ end of the *ashA* gene, there is a putative rho-independent transcription termination sequence and a potential ribosome-binding region. These sequences are followed by an initiation codon and the first 54 nucleotides of an incomplete *ashB* gene (Fig. 5). The first 13 amino acids deduced from this nucleotide sequence are identical to those reported for the same region in *A. pleuropneumoniae* (8). The next 15 nucleotides are completely different from the *A. pleuropneumoniae* sequence and are followed by a stop codon. There are several stop codons in all three reading frames beyond this point in the sequence.

This abrupt termination in the coding sequence was unexpected, since, in all of the RTX determinants characterized previously, the four genes required for the production, activation, and secretion of the toxin were contiguous (12, 14, 18, 24, 40). Recently, a similar discontinuity in the hemolysin determinant was described for *A. pleuropneumoniae* serotypes 7 and 9 (1, 38). In *A. suis*, the interruption was not caused by a cloning artifact, since recloning the DNA fragment containing this region gave similar results and the restriction site used for cloning (*Hin*dIII) was not located near the stop codon.

Since *A. suis* can secrete hemolysin into the growth medium, we presumed that it must possess a secretion mechanism, most likely one similar to that of other RTX toxins. Although the predicted *ashB* and *ashD* genes were not found downstream of the *ashC* and *ashA* genes, they may have been present elsewhere on the chromosome. A 2.3-kbp *Xba*I DNA fragment from the *P. haemolytica lkt* determinant, encompassing the 3′ end of the *lktB* gene and the 5′ end of the *lktD* gene (40), was used to probe the *A. suis* chromosome. Positively hybridizing regions were detected; one of these, a 5.0-kb *Hin*dIII fragment, was cloned into pBR322. Presumably, this clone contains all or a portion of the *ashB* and *ashD* genes, and it is being characterized by restriction endonuclease mapping and DNA sequencing.

DISCUSSION

We have characterized an *A. suis* 3714 hemolysin that is immunologically and genetically related to the previously described RTX cytolysins from *E. coli*, *P. haemolytica*, and *A. pleuropneumoniae*. Unlike previously characterized cytolysins found in separate species, which have had only partial DNA and amino acid homology to one another (7, 12, 14, 24, 27), the deduced amino acid sequence of this protein was highly (99 to 100%) similar to those of the AppA/ClyII proteins of *A. pleuropneumoniae* serotypes 5 and 9 (7, 38). Another unusual feature of the *A. suis* RTX cytolysin determinant is the unexpected discontinuity of the four genes required for the production and secretion of active

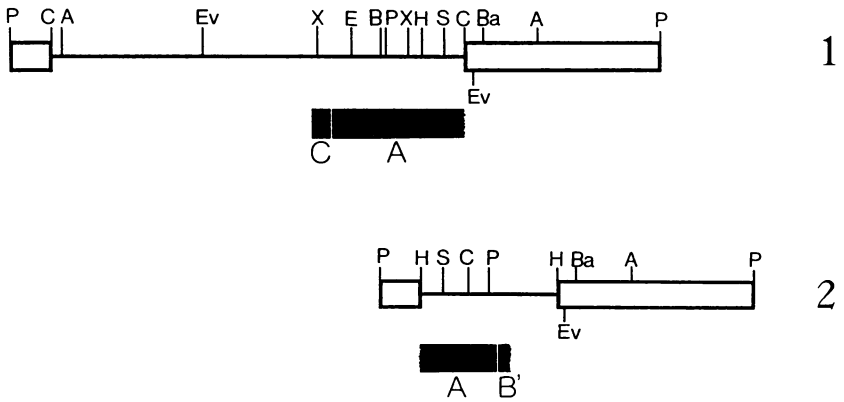

FIG. 3. Restriction endonuclease map of recombinant plasmids containing the *ashC/A* determinant. Plasmid 1 (p3714G) is pBR322 (▭) containing a 7.5-kbp *Cla*I insert with *ashC* and ~80% of *ashA* (■). Plasmid 2 (pHC3-1) is pBR322 containing a 2.5-kbp *Hin*dIII insert with the remainder of *ashA* and a partial *ashB* gene (*ashB′*). These plasmid inserts were subcloned and joined in pBR322 to form plasmid pAsAC-61 (not shown). Abbreviations: A, *Acc*I; Ba, *Bam*HI; B, *Bgl*II; C, *Cla*I; E, *Eco*RI; E_v, *Eco*RV; H, *Hin*dIII; P, *Pst*I; X, *Xba*I.

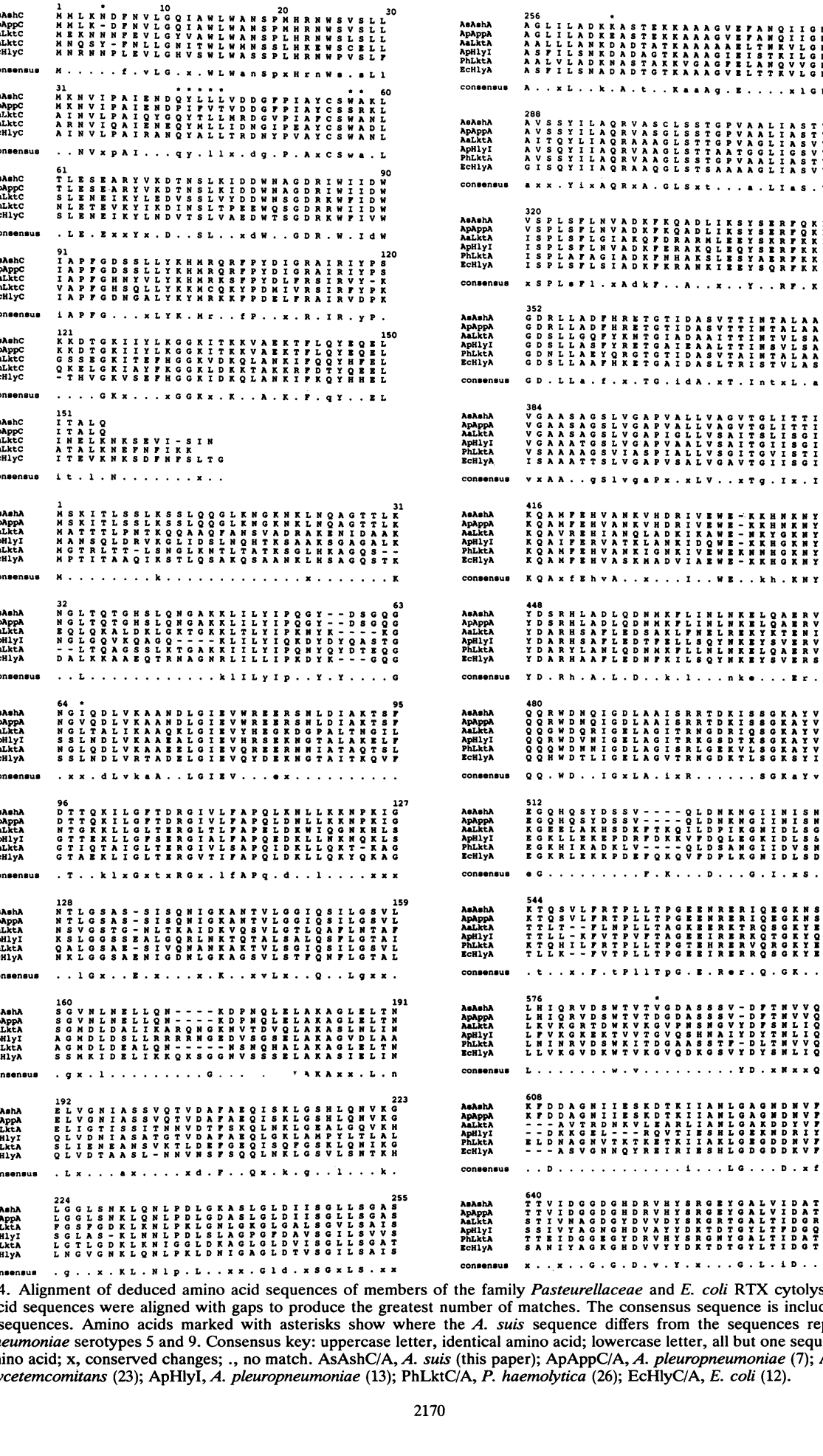

FIG. 4. Alignment of deduced amino acid sequences of members of the family *Pasteurellaceae* and *E. coli* RTX cytolysins. Deduced amino acid sequences were aligned with gaps to produce the greatest number of matches. The consensus sequence is included below the aligned sequences. Amino acids marked with asterisks show where the *A. suis* sequence differs from the sequences reported for *A. pleuropneumoniae* serotypes 5 and 9. Consensus key: uppercase letter, identical amino acid; lowercase letter, all but one sequence have the same amino acid; x, conserved changes; ., no match. AsAshC/A, *A. suis* (this paper); ApAppC/A, *A. pleuropneumoniae* (7); AaLktC/A, *A. actinomycetemcomitans* (23); ApHlyI, *A. pleuropneumoniae* (13); PhLktC/A, *P. haemolytica* (26); EcHlyC/A, *E. coli* (12).

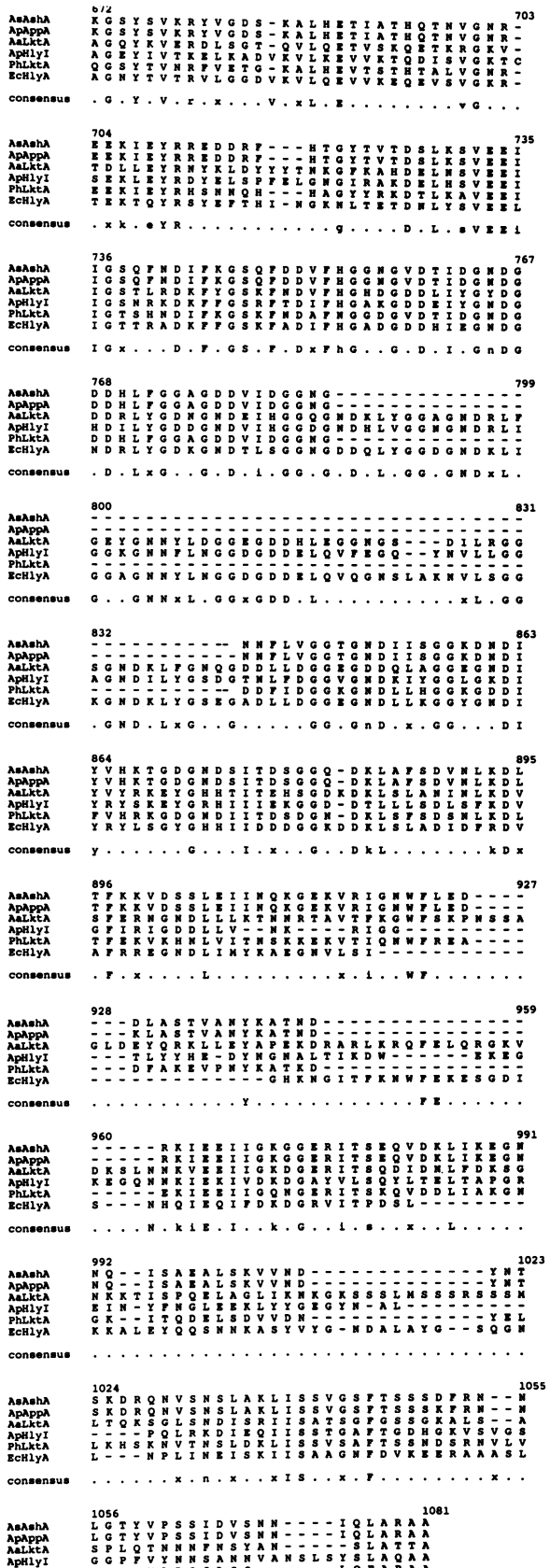

Fig. 4—*Continued.*

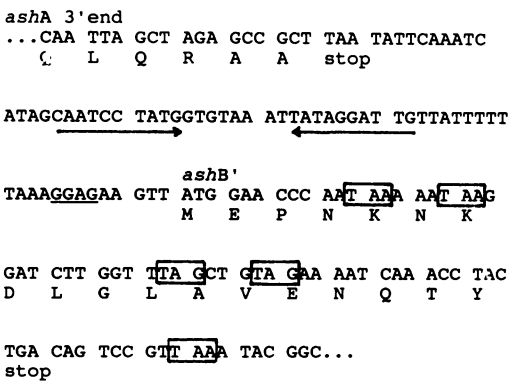

FIG. 5. DNA sequence of *ashA* 3′ end and *ashB′*. After the termination codon at the end of *ashA*, there is a rho-independent transcriptional terminator (arrows) and a potential ribosome-binding site (underlined). *ashB′* encodes only 18 amino acids followed by a stop codon. The first 13 amino acids are identical to those encoded by *A. pleuropneumoniae appB′* (8), whereas the last five are completely different. The stop codons in the other possible reading frames are boxed.

toxin. In most other RTX determinants, the four genes adjoin one another on the same strand of DNA. In *A. suis*, the *ashC* and *ashA* genes are located on a region of the chromosome that is separate from that of the *ashB* and *ashD* genes. A similar interruption was recently detected in the RTX determinants of *A. pleuropneumoniae* serotypes 5, 7, and 9 (1, 8, 38). There is some discrepancy in the location of termination of the partial B gene (pseudogene [8]) in *A. suis* 3714 and *A. pleuropneumoniae* serotype 9 versus that reported for *A. pleuropneumoniae* serotype 5. Chang et al. reported that the *appB* pseudogene (*appBx*) contains the first 167 of an expected 700 codons (8). In contrast, the *A. suis ashB* contains only 17 codons followed by numerous stop codons. The sequence downstream of codon 13 shows no homology to *appBx*. This partial *A. suis* B gene is identical to that of the *A. pleuropneumoniae* serotype 9 determinant (38). The DNA sequence of the hemolysin of *A. pleuropneumoniae* serotype 1 has also been determined (14), but it is not known whether the reported flanking *hlyB*-like open reading frame is complete. Chang et al. reported that, whereas the *appC/A* genes are more similar to the *lktC/A* genes of *P. haemolytica* A1, the *appB/D* genes are more similar to the *hlyB/D* genes of *E. coli* (8). The *ashB* and *ashD* genes from *A. suis* have been cloned and are being characterized. Determination of the DNA sequence of the *ashB/D* genes will permit assessment of their relatedness to those of other RTX determinants (8, 12, 16, 17, 40).

The level of similarity between AppA, HlyIIA, and AshA suggests they diverged only recently. The unusual discontinuity in the hemolysin determinants of these organisms supports the possibility of their common ancestry. Investigating the acquisition of the cytolysin determinants may assist in interpreting the evolutionary relationship between *A. suis* and *A. pleuropneumoniae*. The potential exchange of genetic material between these closely related species is not unlikely, since they share the same host (10, 30). Recently, the cytolysin determinant of *A. pleuropneumoniae* serotype 7 was shown to be flanked by long identical direct repeats, which could lead to the excision of the region through homologous recombination (2). Also, it is well known that the cytolysin determinant of *E. coli* can be encoded both chromosomally and on plasmids (18). These results imply

possible mobility of these cytolysin determinants. On the other hand, both *Actinobacillus* species have identical hemolysin determinants in which the four genes are divided between at least two separate loci (in contrast to other members of the RTX family). Because of the similarity of this split-determinant arrangement, it is improbable that both species acquired the determinants separately. More likely, one of these species was derived from the other with the attendant loss or gain of the requirement for NAD that characterizes *A. pleuropneumoniae* (32). Until recently, *A. pleuropneumoniae* was classified as a member of the genus *Haemophilus* based on its growth requirement for NAD; later, based on DNA homology and phenotypic characteristics, it was judged to be more closely related to the actinobacilli (34). The high degree of similarity between the hemolysin determinants of these two species lends support to this assignment.

Smits et al. recently determined that many serotypes of *A. pleuropneumoniae* produce from one to three types of RTX hemolysins that are only partially homologous to one another and have different biological activities (38). These molecular data confirmed earlier work by Kamp and van Leengoed (21). Smits et al. characterized the ClyII hemolysin, which is identical to the Ash hemolysin, as being only weakly hemolytic compared with the ClyI hemolysin (38). However, *A. suis* is strongly hemolytic and therefore may also produce more than one hemolysin; this possibility is under investigation. In Fig. 1b, the weaker hybridization signals could represent such other RTX hemolysin determinants. Preliminary data showed that, when the *ash* genes were used as probes, only one major hybridization band of approximately 12 kb was detected against chromosomal DNA from the *Actinobacillus* isolates examined (data not shown).

The hemolysin of *A. pleuropneumoniae* has been shown to be one of its more significant virulence factors (9, 19, 20, 35). Animals with high anti-hemolysin titers have been shown to be protected from challenge with *A. pleuropneumoniae* (9). A mutant strain of *A. pleuropneumoniae* serotype 5 that was unable to secrete the hemolysin was much less virulent in mice and pigs and was unable to elicit the production of antibodies that protected against challenge with the parent strain (19). Although the hemolysins produced by *A. suis* and *A. pleuropneumoniae* are homologous, the organisms differ markedly in their ability to cause disease. *A. pleuropneumoniae* is highly virulent and causes acute hemorrhagic pleuropneumonia (3, 30), whereas *A. suis* is an opportunistic pathogen, most often causing septicemia (especially in young animals) and abortion (25, 30). Obviously, other virulence factors besides the cytolysins are involved in the localization and the progression of disease caused by these two species.

Because the cytolysins of these two organisms are similar, it may be possible to use the cloned cytolysin of *A. suis* as a component of a vaccine to prevent pleuropneumonia. *A. suis* and *A. pleuropneumoniae* have been shown to share outer membrane epitopes (29, 35). These common antigens can cause false-positive results when swine herds carrying *A. suis* are screened for carriers of *A. pleuropneumoniae* (36, 37). Epitopes of potential importance in a subunit vaccine could be identified by screening with convalescent swine sera and combined with the cloned *A. suis* cytolysin. In addition, *A. suis* may be suitable as a host for the expression of cloned putative *A. pleuropneumoniae* virulence factors.

Two other species of *Actinobacillus*, *A. equuli* and *A. lignieresii*, possess DNA with homology to the RTX genes (31; this study) but do not appear to express this information in the same manner as overtly hemolytic or leukotoxic species. In *A. actinomycetemcomitans*, both leukotoxic and nonleukotoxic strains possess RTX determinants, but their levels of toxin production differ. Leukotoxic strains produce much higher levels of *lkt* mRNA than do nonleukotoxic strains (39). Proteins with similarity to RTX cytolysins were not detected in log-phase culture supernatants from *A. equuli* or *A. lignieresii*, either on SDS-PAGE gels or on Western blots. However, proteins of approximately 105 to 110 kDa that reacted with antiserum against AppA were detected in early-stationary-phase culture supernatants (5). Therefore, these *Actinobacillus* species appear to express the RTX genes to some extent. The genetic organization of the RTX loci of these species is now being studied to further elucidate the distribution of these determinants in species of the genus *Actinobacillus*.

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