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The *Actinobacillus pleuropneumoniae* Hemolysin Determinant: Unlinked *appCA* and *appBD* Loci Flanked by Pseudogenes

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The *appBD* genes encoding the secretion functions for the 110-kDa RTX hemolysin of *Actinobacillus pleuropneumoniae* have been cloned and sequenced. Unlike analogous genes from other RTX determinants, the *appBD* genes do not lie immediately downstream from the hemolysin structural gene, *appA*. Although isolated from a diverse group of gram-negative organisms, the *appBD* genes and the characterized RTX *BD* genes from other organisms all exhibit a high degree of homology at both the DNA and predicted amino acid sequence levels. Analysis of the DNA sequences 3′ to *appA* and 5′ to *appB* suggests that these regions harbor remnant RTX *B* and *A* pseudogenes, respectively. Although the *appA* gene is most similar to the *lktA* gene from *Pasteurella haemolytica* (Y. F. Chang, R. Young, and D. K. Struck, DNA 8:635–647, 1989), the RTX *A* pseudogene upstream from *appB* most closely resembles the *hlyB* gene from *Escherichia coli*, suggesting that the *appCA* and *appBD* operons were derived from different ancestral RTX determinants.

Porcine pleuropneumonia is a highly contagious respiratory disease caused by *Actinobacillus pleuropneumoniae* and is a major cause of economic loss to the swine industry (25). Since sterile culture supernatants from *A. pleuropneumoniae* induce a localized pneumonia similar to that seen in naturally infected pigs (28), it is probable that one of the virulence factors of *A. pleuropneumoniae* is a secreted cytotoxin. A likely candidate for this extracellular toxin is the 110-kDa hemolysin produced by pathogenic serotypes of *A. pleuropneumoniae* (5).

The gene for the 110-kDa hemolysin has been cloned, and DNA sequence analysis indicates that it belongs to the RTX family of cytotoxins (4), a family which includes the hemolysins produced by *Escherichia coli* (6, 11), *Proteus vulgaris* (15), and *Morganella morganii* (15) and the leukotoxins produced by *Pasteurella haemolytica* (13, 19) and *Actinobacillus actinomycetemcomitans* (17, 18). Analysis of the genetic determinants for secreted RTX toxins has revealed a common structure consisting of four linked genes, *CABD*, expressed from a promoter upstream of the *C* gene (14, 32, 35). The *A* gene encodes the secreted toxin protein, and the *C* gene product provides a function necessary for the efficient conversion of the A protein into its biologically active form either prior to or during the secretory process (7, 24). The mechanism by which the A protein is converted into its active form is unknown, but monoclonal antibodies which recognize only the HlyC-activated form of HlyA have been reported (27). The RTX *BD* genes are required for toxin secretion (8, 16, 20), a process which is also not understood at the molecular level.

The predicted amino acid sequence of RTX B proteins suggests the existence of two domains: a hydrophobic N-terminal domain believed to direct localization of the protein to the bacterial inner membrane, and a C-terminal domain containing two potential nucleotide-binding sites which are highly conserved in the B proteins from *E. coli* (6, 11), *P. vulgaris* (16), *P. haemolytica* (13, 31), and *A. actinomycetemcomitans* (9). The first nucleotide-binding site is common to a number of prokaryotic and eukaryotic proteins involved in the membrane transport of small molecules (1, 12). The protein from the second binding site is highly variable and is not strongly homologous to any kinase of any prokaryotic or eukaryotic organism (16). RTX *D* gene homologs have been found only in systems which function to transport proteins across membranes; transport proteins for small molecules share sequence similarity only to RTX B proteins. It has recently been reported that in the case of *E. coli* alpha-hemolysin, the *BD* gene products are not sufficient for hemolysin secretion. At least one gene which is not part of the *hly* locus, that for the outer membrane protein TolC, is also required for this process (34). Thus, the transport of RTX A proteins across the bacterial envelope may require a secretory apparatus consisting of more than just the *BD* gene products.

In a previous report (4), we presented the complete nucleotide sequence of the *A. pleuropneumoniae C* and *A* genes and noted that the 164 bp of sequence immediately downstream from *appA* contained a potential rho-independent transcriptional terminator found in other RTX operons. This terminator was followed by a Shine-Dalgarno sequence and an open reading frame identical to that of the *lktB* gene from *P. haemolytica* in 26 of the 36 reported codons. Subsequent analysis has shown that the *B* gene-like sequences which lie 3′ to *appA* represent an incomplete or remnant RTX *B* pseudogene. Intact *appBD* genes have been found elsewhere on the *A. pleuropneumoniae* chromosome, unlinked to *appCA*. This is the first report that the four genes which make up the typical RTX determinant are not present in the same transcriptional unit.

MATERIALS AND METHODS

Bacterial strains, vectors, plasmids, and growth conditions. *A. pleuropneumoniae* serotypes 1 to 7 were gifts of C. Pijon, University of Minnesota, St. Paul. Serotypes 8 to 12 were

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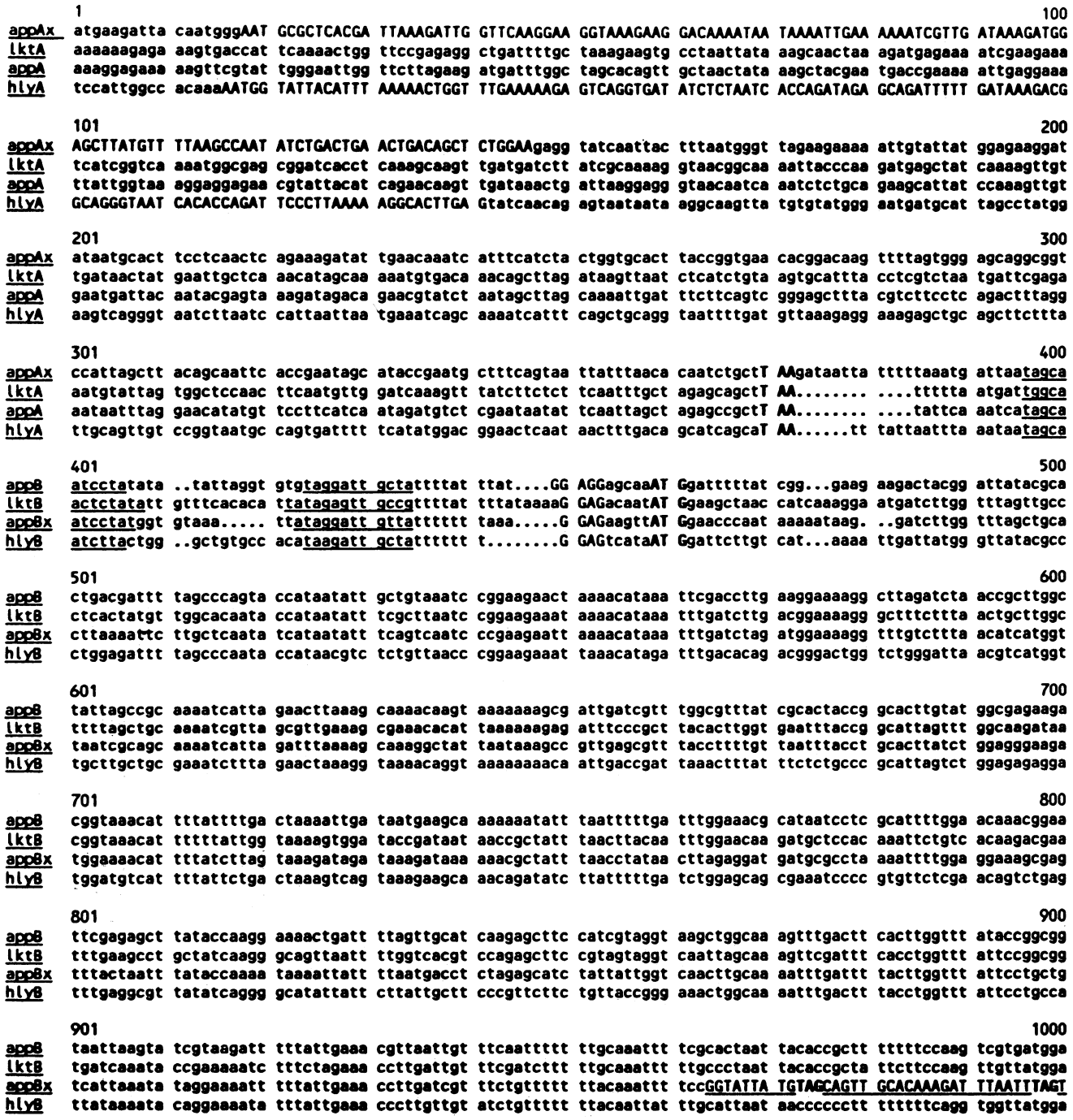


FIG. 1. Comparison of the nucleotide sequences 5′ and 3′ to the *appAx-appB*, *lktA-lktB*, *appA-appBx*, and *hlyA-hlyB* intergenic regions. The termination and initiation codons for the *A/B* genes/pseudogenes are aligned at positions 370 and 459, respectively. Potential rho-independent terminators in the intergenic regions are underlined (positions 396 to 434). The point at which the *appBx* sequence diverges from the consensus *B* sequence is at position 964, and the sequence for *appBx* beyond this point is underlined; two in-frame stop codons in this divergent sequence are indicated in boldfaced type. The capitalized positions for *appAx* (positions 18 to 145) and *hlyA* (positions 15 to 141) yield similar predicted amino acid sequences (see Fig. 5) upon translation if shifts in the *appAx* reading frame are permitted.

gifts of K. R. Mittal, University of Montreal, Saint-Hyacinthe, Quebec, Canada, and were grown in brain heart infusion broth supplemented with 0.1% NAD. All *E. coli* strains [JM101, *supE thi* Δ(*lac-proAB*) F′(*traD36 proAB*$^+$ *lacI*^{q} *lacZ*Δ*M15*); TB1, *ara* Δ(*lac-proAB*) *rpsL* ϕ80d*lacZ*Δ *M15 hsdR17* (r^- m^+); LE392, *hsdR514* (r^- m^+) *supE44 supF58 lacY1 galK2 galT22 metB1 trpR55*; and P2392, a P2 lysogen of LE392] were cultured in Luria broth (22) containing the appropriate antibiotics when necessary. The vectors Lambda-Dash, M13mp18, M13mp19, and pHG165 were as previously described (4). The p15a-based plasmid vector, pAC8, was constructed by partial *Hae*II digestion of *Pst*I-

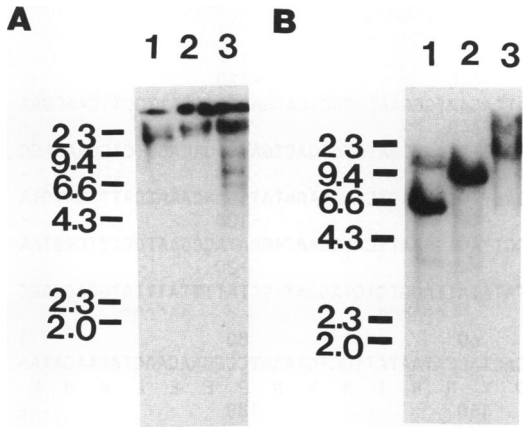


FIG. 2. The *appBD* genes are not linked to *appCA* on the *A. pleuropneumoniae* chromosome. *A. pleuropneumoniae* genomic DNA was digested with either *Eco*RI (lane 1), *Bam*HI (lane 2), or *Xho*I (lane 3). Hybridization probes for the Southern blots are described in Materials and Methods. (A) Hybridization with a probe specific for the RTX *CA* genes. (B) Hybridization with a probe specific for the RTX *BD* genes.

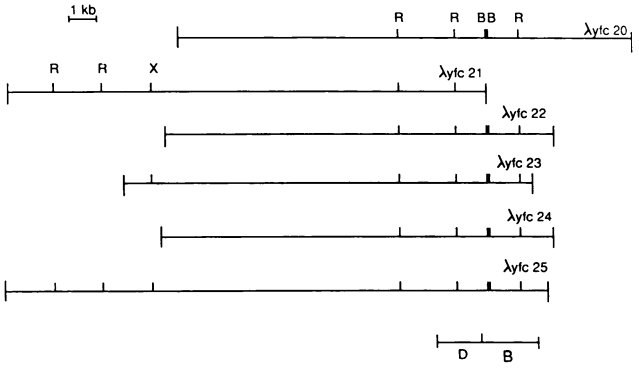


FIG. 3. Restriction maps of the *A. pleuropneumoniae BD* gene clones. All of the clones except λyfc21 contain the complete *BD* gene sequences. The locations of the two open reading frames designated *appB* and *appD* are indicated. The *Eco*RI sites which flank the inserts are derived from the vector. R, *Eco*RI; B, *Bam*HI; X, *Xba*I.

digested pACYC177 (2). The DNA was converted into blunt-ended fragments with DNA polymerase and religated. The religated DNA was digested with *Hae*II and mixed with the small *Hae*II fragment of pUC8 (33), which contains the *lac* control elements, the *lacα* gene fragment, and the polylinker cloning sites.

The *appA* and *appCA* genes were subcloned from λyfc7 (4) and λyfc8 (4) into pAC8 as *Eco*RI-*Sal*I fragments to form the plasmids pYFC66 and pYFC67. The intact *appBD* genes from λyfc25 were subcloned into pHG165 as a *Sal*I fragment to form pYFC65. ColE1-based plasmids expressing the *appA* (pYFC38) and *appCA* (pYFC37) genes have been previously described (4). pYFC35 (3) and pLG575 (20) were used as sources for the expressed *P. haemolytica lktBD* and *E. coli hlyBD* genes, respectively.

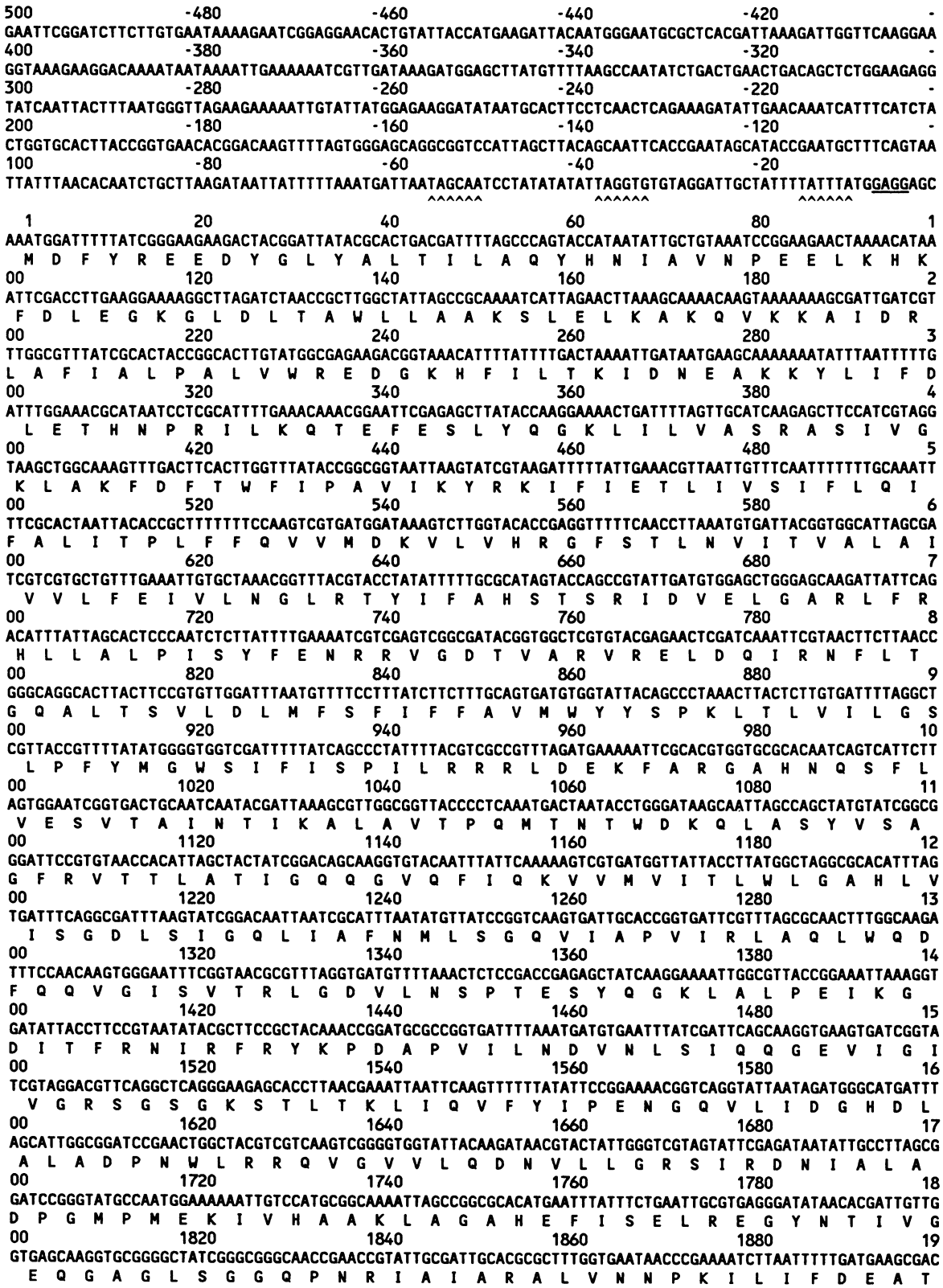
Probes for Southern blotting and library screening. A 3-kb *Eco*RI-*Cla*I fragment from pYFC19 (3) was used to detect sequences homologous to RTX *CA* genes. This probe is derived from the *lkt* determinant of *P. haemolytica* and contains 67 bp upstream from the *lktC* gene, the entire *lktC* sequence, and the first 2,302 bp of the *lktA* gene. A 2.5-kb *Xba*I fragment from pYFC32 (3) was used to detect sequences homologous to RTX *BD* genes. This probe contains 1,657 bp from the 3′ end of *lktB* and 511 bp from the 5′ end of *lktD*. The relevant fragments were purified by agarose gel electrophoresis and labeled with [α-^{32}P]dATP by nick translation. The plasmid pYFC37 (4) was used as the source of the *appCA* genes.

Southern blotting and library screening. *A. pleuropneumoniae* genomic DNA was prepared by the method of Silhavy et al. (30), digested with *Bam*HI, *Eco*RI, or *Xho*I, electrophoresed through a 0.7% agarose gel, and transferred to nitrocellulose. The previously described *A. pleuropneumoniae* genomic library in the bacteriophage vector Lambda-Dash (4) was plated on 150- by 15-mm plates at a density of 1,000 plaques per plate, and the plaques were transferred to nitrocellulose filters. Filters were hybridized in 6× SET (20× SET is 3 M NaCl, 20 mM EDTA, and 0.4 M Trizma base, pH 7.8)–5× Denhardt's solution–100 μg of sheared calf thymus DNA per ml–50 μg of polyadenylic acid per ml for 12 h at 50°C. They were then washed with 4× SET at room temperature and then once each with 4× SET, 2× SET, and 1× SET at 50°C, after which they were subjected to autoradiography. For library screening, plaques which gave positive signals were picked, rescreened, and amplified on P2392.

DNA sequencing and analysis. DNA sequencing was performed by the dideoxy-chain termination method (29). Regions from the *A. pleuropneumoniae* insert in bacteriophage clone λyfc25 were subcloned into M13mp18 or M13mp19, and single-stranded phage DNA was prepared by standard procedures (21). In the sequencing reactions we used [^{35}S]dATP, T7 DNA polymerase, and the commercially available Sequenase kit (United States Biochemicals, Cleveland, Ohio). Certain regions of the DNA insert were sequenced directly from the recombinant bacteriophage. In these cases, 1 to 2 μg of λyfc25 DNA was mixed with 100 ng of an oligonucleotide primer (prepared by the Analytical and Synthetic Facility, Cornell University) in a total volume of 12 μl, and the mixture was boiled for 5 min and cooled rapidly on ice. The sequencing reactions were performed with reagents supplied with the Sequenase kit as specified by the manufacturer. DNA sequence analysis was performed on a VAX computer by using the Genetics Computer Group program package (University of Wisconsin, Madison) and programs from the PC Gene package (IntelliGenetics Corp., Mountain View, Calif.). The amino acid sequence alignment was carried out with the GAP and LINEUP programs (Genetics Computer Group, University of Wisconsin), and similarity was calculated by the method of Pearson and Lipman (26).

SDS-PAGE and Western blotting. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western immunoblotting were performed as previously described (3) by using culture supernatants (5 ml) concentrated by the chloroform-methanol-water system (36) and resuspended in 150 μl of sample buffer. After boiling for 2 min, 1 μl of sample from *A. pleuropneumoniae* and 25 μl of sample from *E. coli* TB1 harboring *app* were subjected to SDS-PAGE. Immunoreactive proteins were visualized by using porcine antihemolysin (4) and an anti-swine immunoglobulin G second antibody conjugated to alkaline phosphatase.

Nucleotide sequence accession number. The sequence data presented in this paper have been submitted to GenBank and assigned the accession number M65808.



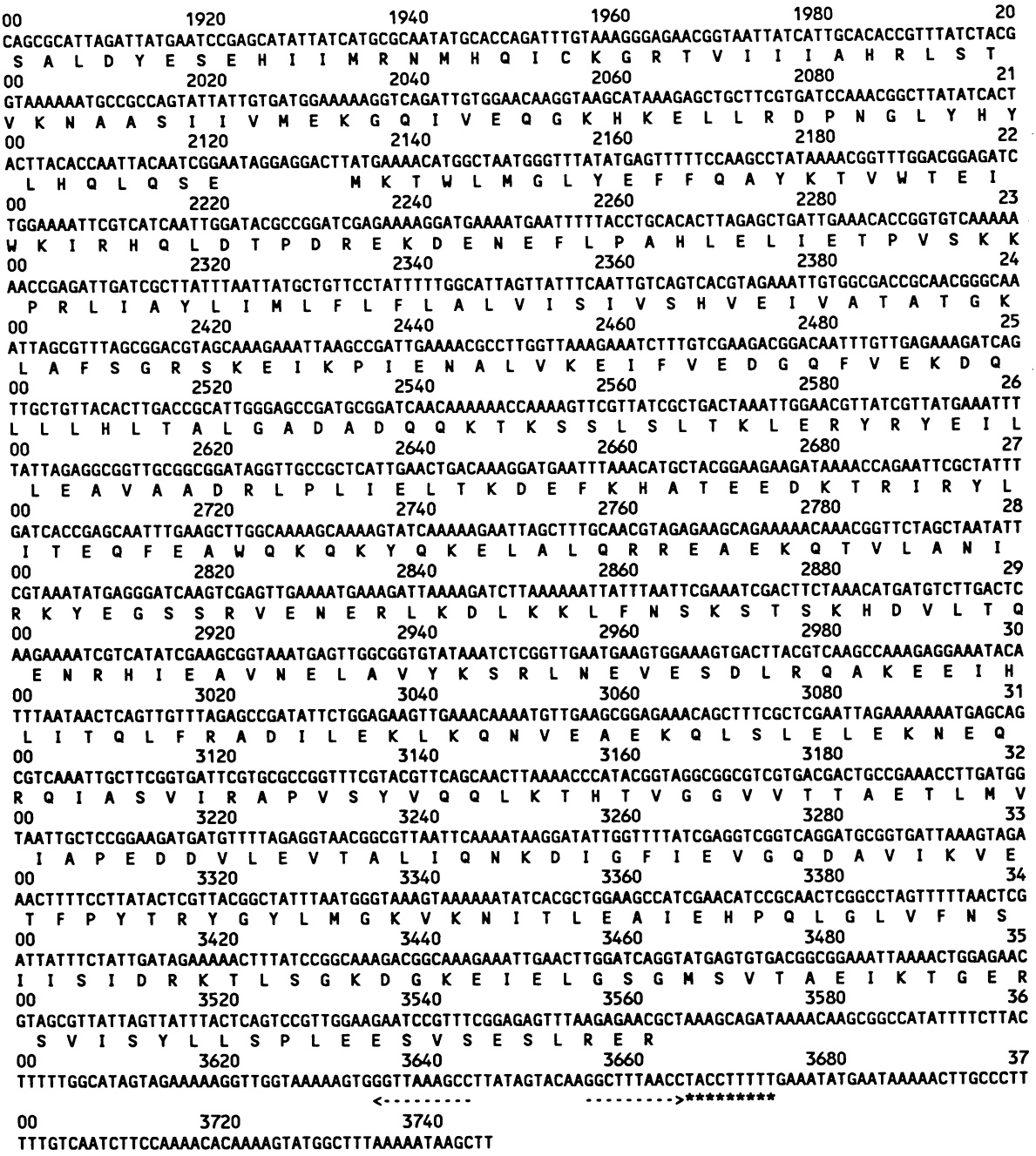


FIG. 4. Nucleotide sequence of the *appBD* region and the predicted amino acid sequences of the AppB and AppD proteins. Promoterlike regions proximal to *appB* and *appD* are underlined. Potential ribosome-binding sites preceding *appB* and *appD* are indicated by ^^^^^^. A potential rho-independent transcription terminator and poly(T) track distal to *appD* are indicated by >----< and ****, respectively.

RESULTS

The *appCA* locus does not contain functional *appBD* secretion genes. We previously reported the complete nucleotide sequence of the *appCA* hemolysin genes from *A. pleuropneumoniae* serotype 5 (4). The sequence extending 172 bp to an *Xba*I site downstream of the termination codon of *appA* contained the hallmarks of the RTX *A-B* intergenic region described for the *E. coli* hemolysin (35), the *A. actinomycetemcomitans* leukotoxin (9), and the *P. haemolytica* leukotoxin (13, 31) loci; namely, a rho-independent transcriptional terminator and a canonical Shine-Dalgarno sequence for the downstream *B* gene. Moreover, the open reading frame for the putative *B* gene extended for 36 codons, of which 26 were identical to the *lktB* gene of *P. haemolytica* (4). On the basis of these results, we reasonably expected that functional *appBD* genes homologous to *hlyBD*/*lktBD* would lie immediately downstream of *appA*. Initial sequence analysis beyond the *Xba*I site was consistent with this expectation since the next 131 codons in the linear sequence also showed significant similarity to the known

TABLE 1. Characteristics of the AppB and AppD proteins and their genes

App gene or protein	Homology to: *P. vulgaris* hemolysin locus		*E. coli* J96 hemolysin locus		*P. haemolytica* leukotoxin locus		*A. actinomycetemcomitans* leukotoxin locus		$10^3 M_r$	pI
	Nucleotide[a]	Amino acid[b]	Nucleotide	Amino acid	Nucleotide	Amino acid	Nucleotide	Amino acid		
B	76.0	92.0	73.4	90.3	76.1	91.4	72.7	89.5	79.4	9.70
D			65.4	78.7	68.4	81.5	64.0	77.1	54.2	5.67

[a] Percent identical residues.
[b] Percent identical residues assuming that the following amino acid pairs are equivalent: I and V; S and T; E and D; K and R; F and Y.

RTX *B* genes (Fig. 1). Surprisingly, the DNA sequence beyond this point immediately diverged from those of *hlyB*/*lktB*, with multiple stop codons appearing in all three reading frames (Fig. 1 and data not shown). No further homology to *hlyBD or lktBD* was found in the approximately 8,000-bp region downstream of the point of divergence by DNA sequencing or Southern blotting with probes derived from the 3′ end of *lktB* or from *lktD* (data not shown). A cloning artifact was ruled out since we identified nine overlapping clones with internally consistent restriction maps when using both DNA and antibody probes (4). In addition, the point at which the sequence divergence occurs is not located at a potential *Sau*3A site, the enzyme used for the partial digestion of the DNA that was used for the construction of the genomic *A. pleuropneumoniae* library. Consequently, the sequence that we originally concluded to belong to *appB* actually represents a pseudogene located immediately downstream of *appA*. This pseudogene, designated *appBx*, consists of 167 codons related to the N termini of known RTX B proteins (the expected *B* gene should code for a protein of approximately 700 residues).

Identification of RTX *BD* gene sequences unlinked to *appCA*. Since it had been demonstrated that the expression of *hlyBD* in *trans* to *appCA* confers a hemolytic phenotype on *E. coli* (2a, 10), it seemed likely that the secretion of AppA by *A. pleuropneumoniae* would occur by a mechanism involving related *appBD* genes. To determine whether RTX *BD*-like sequences other than *appBx* existed in the *A. pleuropneumoniae* genome, we performed genomic Southern blots with a probe derived from *lktBD*. This probe contained sequences from the distal portion of *lktBD* and thus did not contain homology to *appBx*. As can be seen in Fig. 2B, a 6.5-kb *Bam*HI fragment and a 9-kb *Eco*RI fragment of *A. pleuropneumoniae* DNA hybridized to this probe. In contrast, all genomic sequences hybridizing to an RTX *CA* gene-specific probe remained in high-molecular-weight DNA (Fig. 2A). This suggested that intact and functional *appBD* genes might be unlinked to *appCA* in the *A. pleuropneumoniae* genome.

To find whether other serotypes (serotypes 1 to 12) and other isolates of serotype 5 also showed the same hemolysin operon arrangement in this species, we performed Southern blots of their genomic DNAs which were cut by *Hin*dIII. However, owing to the restriction enzyme polymorphism of other serotypes and two other isolates of serotype 5, it is difficult to conclude that all serotypes in this species share the same hemolysin operon arrangement (data not shown).

Cloning of the *appBD* locus. We next screened an *A. pleuropneumoniae* library in the vector Lambda-Dash (4) with the same distal *lktBD*-specific probe to identify clones with RTX *BD*-like sequences. A series of overlapping clones (Fig. 3) distinct from the *appCA* clones previously described (4) were isolated. Sequence analysis of bacteriophage clone λyfc25 revealed that it carries intact RTX *BD* gene homologs which we designate *appBD* (Fig. 4). Southern blotting demonstrated that none of the bacteriophage clones carrying the *appBD* genes contained regions homologous to *appCA* (data not shown).

The *appBD* sequence was examined for *E. coli* promoter-like sequences by using the homology score method (26). There were a sequence similar to the TATTTA consensus promoter sequence (−10 region) and two sequences, TTAGGT and TTAATA, similar to the consensus RNA polymerase-binding site (23) proximal to *appB* (Fig. 4). Upstream of the start codon of *appB* is a potential ribosome-binding site (Fig. 4). A ribosome-binding site and a promoter sequence with consensus −10 and −35 regions lie proximal to *appD* (Fig. 4). A sequence very similar to the rho-independent transcriptional terminator of *E. coli* downstream from *appD* was also observed (Fig. 4). Table 1 summarizes the similarities between *appBD* and the other RTX *BD* genes for which sequence information is available.

An RTX *A* remnant pseudogene lies upstream from *appBD*. Examination of the region immediately upstream of the *appB* gene reveals sequences strikingly similar to the intergenic region separating the *A* and *B* genes of other RTX loci, including the presence of a potential rho-independent terminator (Fig. 1). Further upstream, at the position occupied by the 3′ ends of the *A* genes in other RTX loci, all three possible reading frames contain numerous stop codons, indicating the absence of a functional *A* gene. However, allowing for the accumulation of frameshift mutations, the amino acid sequences predicted by the three interrupted reading frames can be combined to yield one sequence which shows significant similarity to HlyA but not LktA (Fig. 5), suggesting that this region comprises an RTX *A* pseudogene which we designate *appAx*. Thus, it appears that the intact *appA* and *appB* genes lie adjacent to 3′-*B* and 5′-*A* pseudogenes, respectively.

The *appBD* genes function in *E. coli*. To demonstrate that the newly identified *appBD* genes provided the expected RTX secretion functions, we subcloned the entire insert of λyfc25 into the plasmid vector, pHG165, as a *Sal*I fragment to form pYFC65. When pYFC65 was introduced into *E. coli* harboring the *appCA* genes on a compatible plasmid, the

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___NALTIKDWFKEGKE_GQ__NNKIEKIVDKDGAYVLSQYLTELTALE_______ (appAx)
___NGITFKNWF_E_KESGDISNHQIEQIFDKDGR_VITPDSLKK_ALE_______ (hlyA)
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FIG. 5. Comparison of the *appAx* pseudogene with *hlyA*. The sequences aligned correspond to codons 906 to 947 of the *hlyA* gene (6, 11) and an amino acid sequence derived from nucleotides 18 to 145 of the *appAx* sequence shown in Fig. 1.

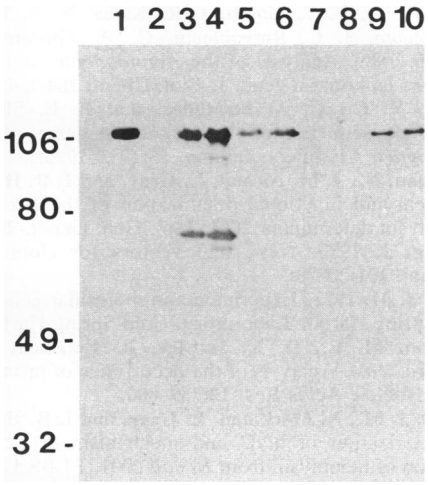

FIG. 6. The *appBD* genes function in *E. coli*. Western blot analysis of culture supernatants from *A. pleuropneumoniae* serotype 5 (1 μl applied) and from *E. coli* strain TB1 harboring combinations of plasmids containing genes cloned from RTX loci (25 μl applied). Lanes: 1, *A. pleuropneumoniae*; 2, TB1 with pHG165 and pAC8 (vectors); 3, TB1 with pYFC66 (*appA*) and pYFC35 (*lktBD*); 4, TB1 with pYFC67 (*appCA*) and pYFC35; 5, TB1 with pYFC37 (*appCA*) and pLG575 (*hlyBD*); 6, TB1 with pYFC38 (*appA*) and pLG575; 7, TB1 with pYFC66; 8, TB1 with pYFC67; 9, TB1 with pYFC66 and pYFC65 (*appBD*); 10, TB1 with pYFC67 and pYFC65. Prestained molecular mass markers were from Bio-Rad Laboratories and are nominally 106, 80, 49.5, and 32.5 kDa. Lower-molecular-mass immunoactive bands are ascribed to proteolytic degradation of the full-length toxin.

AppA protein was found to be secreted from the bacteria (Fig. 6). In the absence of the *BD* gene functions provided in *trans* by pYFC65, the AppA hemolysin accumulated intracellularly and was not detected in cell-free culture supernatants. As expected, expression of the *lktBD* genes from *P. haemolytica* and the *hlyBD* genes from *E. coli* in *trans* to *appCA* also allowed secretion of the AppA hemolysin (Fig. 6).

DISCUSSION

Further analysis of previously isolated recombinant bacteriophage clones with *A. pleuropneumoniae* genomic inserts containing the *appCA* genes indicated that intact homologs of RTX *BD* genes were not located distal to *appA* (Fig. 1) as would be predicted from the genetic organization of all other characterized RTX determinants. Since Southern blot analysis suggested that *BD* homologs might exist unlinked to *appCA* (Fig. 2), we rescreened an *A. pleuropneumoniae* genomic library with a hybridization probe derived from *lktBD*. This yielded a series of overlapping clones which proved, by DNA sequence analysis, to contain the intact *appBD* genes (Fig. 3 and 4). These genes were found to direct the secretion of the *A. pleuropneumoniae* hemolysin from *E. coli* expressing *appCA* (Fig. 6). On the basis of the restriction maps of our previously isolated *appCA* clones and the clones carrying *appBD* described in this report, these two loci are separated by a minimum of 8 kb in the *A. pleuropneumoniae* genome.

Previous comparisons have shown that, of the RTX *CABD* genes, the *BD* gene pair are the most conserved while the *C* gene, and, particularly, the *A* genes diverge considerably from organism to organism. Indeed, allowing for conservative amino acid substitutions, the predicted amino acid sequences for the AppB and AppD proteins are between 88–91% and 77–78% identical to the corresponding B and D proteins from *P. vulgaris*, *E. coli*, *P. haemolytica*, and *A. actinomycetemcomitans* (Table 1). Alignment of the predicted amino acid sequences for the five characterized RTX *B* genes indicates that each protein contains two consensus ATP-binding folds which, for AppB, lie between residues 495–513 and 603–625 (Fig. 4).

The finding that the *appBD* genes are not linked to the *appCA* genes represents the first instance in which the RTX genes encoding a secreted toxin are not organized into a single transcriptional unit. Examination of the sequences downstream from *appA* and upstream from *appB* reveals the presence of RTX *B* and RTX *A* pseudogenes, respectively (Fig. 1 and 5). The *appBx* pseudogene shows roughly the same degree of homology to all other RTX *B* genes in pairwise comparisons. This is not surprising, given the fact that the RTX *B* gene is the most highly conserved of the RTX determinants (Table 1). However, the predicted AppA protein is very similar to the leukotoxin from *P. haemolytica* (64% identity [17]) and is less related to the leukotoxin from *A. actinomycetemcomitans* (42% identity [17]) and the hemolysin of *E. coli* (43% identity [17]). This suggests that the *appCABx* locus was derived from a *P. haemolytica lkt*-like determinant. In contrast, the *appAx* pseudogene is most similar to *hlyA* (Fig. 5), suggesting that the *appAxBD* locus originated from an *E. coli hly*-like determinant rather than a *P. haemolytica lkt*-like determinant.

The *appAx* pseudogene appears to have diverged from an *hlyA*-like ancestral gene a considerable time ago, given the accumulation of multiple frameshift mutations. In contrast, the disruption of the *appCABx* locus appears to have been relatively recent since the *appBx* pseudogene is highly homologous to other RTX *B* genes and contains no stop codons or frame shifts. The fate of the *BD* genes, which, we presume, were originally contiguous to *appCA*, is unknown. The abrupt loss of homology to RTX loci which occurs at codon 167 of *appBx* suggests a transposon insertion or deletion event. However, no homologies with known transposable or deletionogenic elements were found anywhere for more than 4 kb downstream of *appBx* (data not shown). In fact, no open reading frames of significant length were found in this region, suggesting that it does not encode any proteins. We conclude that the unlinked *appCABx* and *appAxBD* loci are derived from separate RTX determinants which were introduced into *A. pleuropneumoniae* at different times during the history of this organism.

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