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Isolation and Molecular Characterization of Spontaneously Occurring Cytolysin-Negative Mutants of *Actinobacillus pleuropneumoniae* Serotype 7†

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***Actinobacillus pleuropneumoniae* serotype 7 strains are shown to spontaneously lose cytolytic activity with a frequency of approximately 10^{-4}. The phenotypic change is associated with the loss of approximately 8.5 kbp of chromosomal DNA. A genomic fragment encoding the cytolysin and its flanking sequences was cloned and characterized. Also, the corresponding truncated fragment was cloned from a spontaneous mutant. Comparison of the two clones allowed the definition of the excision site. The ends of the excised fragment are composed of 1,201 bp long direct identical repeats, possibly facilitating the genotypic change by homologous recombination. In accordance with this hypothesis, one repeat is conserved in the spontaneous mutant. Each repeat contains one open reading frame preceded by a Shine-Dalgarno consensus sequence, and the ends of each repeat contain 26-bp complementary sequences with four mismatches.**

Rearrangement of bacterial DNA as a means to increase the fitness of survival of the respective bacterial population is a relatively common phenomenon. Examples are the genetic switch resulting in the expression of an antigenically distinct flagella protein (H-antigen) in *Salmonella* species (25), the on/off switch of type 1 fimbriae in *Escherichia coli* (1), and the chromosomal rearrangements leading to the expression of antigenically distinct fimbriae in *Moraxella bovis* (18) and *Neisseria gonorrhoeae* (23). More recently, the site-specific integration of the virulence plasmid of enteroinvasive *E. coli* and *Shigella flexneri* into the chromosome has been described; this results in a noninvasive and thus avirulent phenotype (36). Also, high-frequency excision of mobile genetic elements under conditions of nutritional stress has been described; this restored the relevant catabolic genes to normal function (8, 24). Insertion and excision events are also encountered in combination with horizontal transfer of virulence factors. Thus, the heat-stable toxins of enterotoxigenic *E. coli* (STI and STII) can be carried by transposable elements, and the inverted repeats in these elements are composed of insertion sequences IS*1* and IS*2*, respectively (11, 15, 27, 28).

The cytolysins or RTX toxins form a large family of calcium-dependent, pore-forming toxins which can be found in different genera of the families *Enterobacteriaceae* and *Pasteurellaceae* (34). Genetic investigation of these toxins has shown that the toxin-encoding gene (*hlyA* in *E. coli*) is commonly clustered with at least three other genes encoding an activator (HlyC) and proteins involved in membrane transport (HlyB and HlyD [5, 35]). A comparison of the nucleotide and amino acid sequences of RTX toxins from different species shows a high degree of similarity, thus suggesting the occurrence of horizontal transfer rather than parallel evolutionary evolvement (2, 5, 6, 16, 34).

In *Actinobacillus pleuropneumoniae* at least three immunologically and functionally distinct cytolysins of $\pm 110 \times 10^3$, $\pm 105 \times 10^3$, and $\pm 103 \times 10^3$ Da (±110K, ±105K, and ±103K proteins, respectively) have been described (12). The distribution of the ±110K and the ±105K proteins is limited to a few serotypes, whereas the ±103K protein has been found in all serotypes with the exception of serotype 10 (12).

In the current communication, we describe the spontaneous loss of the ±103K cytolysin in *A. pleuropneumoniae* serotype 7. We characterize the cytolysin-positive and -negative phenotypes and give a likely explanation for the phenomenon, which could also play a role in the comparatively wide distribution of this protein among different serotypes.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. *A. pleuropneumoniae* AP76 (serotype 7) and AP37 (serotype 1) were isolated from the lungs of diseased pigs submitted to the Western College of Veterinary Medicine, Saskatoon, Sask., Canada. *A. pleuropneumoniae* AP205 (serotype 7) was isolated from a diseased pig in Nebraska and kindly provided by M. L. Chapek, Modern Veterinary Products, Omaha, Neb. *E. coli* NM538 (*supF hsdR*) and NM539 (*supF hsdR* P2cox) served as hosts for the bacteriophage λ library. *E. coli* HB101 (*hsdM hsdR recA*) was used for transformations with plasmid DNA. Plasmid pGH432 was used as an expression vector. It is 4.3 kb in size and encodes an ampicillin resistance determinant and the *lac* repressor. It contains a *tac* promoter followed by unique *Bgl*II, *Sma*I, and *Bam*HI sites, allowing in-frame fusions with an artificial leader peptide and stop codons in all three reading frames. The *A. pleuropneumoniae* genomic libraries were constructed by using the bacteriophage vector λ2001 (Stratagene, La Jolla, Calif.). Plasmid pAA210 (our unpublished results) containing the *Pasteurella haemolytica* leukotoxin gene cluster was used to probe the bacteriophage library. Phages M13mp18 and M13mp19 were grown in *E. coli* JM105. *A. pleuropneumoniae* strains were cultured in PPLO medium (Difco Laboratories, Detroit, Mich.) supplemented with IsoVitaleX (1%, vol/vol [BBL Microbiology Systems, Becton Dickinson and Co., Cockeysville, Md.]). *E. coli* NM538 and NM539

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were grown in CY medium (17) supplemented with 50 mM Tris-HCl (pH 7.5) and 10 mM $MgCl_2$. *E. coli* HB101 and JM105 were grown in Luria medium (17). The concentration of ampicillin used for the growth of pGH432 transformants was 100 mg/liter.

Preparation of DNA and Southern blotting. Genomic DNA was prepared as previously described (29). Plasmid DNA was prepared from chloramphenicol (100 mg/liter)-amplified cultures by alkaline lysis and ethidium bromide-cesium chloride gradient centrifugation (17). Bacteriophage λ2001 DNA was prepared as described by Maniatis et al. (17). All DNA restriction enzyme digests were done in T4 DNA polymerase buffer (17) supplemented with 1 mM dithiothreitol and 3 mM spermidine. Digested DNA was electrophoretically separated on a 0.7% agarose gel and transferred to nitrocellulose by capillary blotting. Probes were labeled with [α-^{32}P]dATP by using random hexanucleotide primers (4). Unincorporated [α-^{32}P]dATP was removed by passage through a Sephadex G-50 column. Filters were prehybridized in 5× Denhardt's solution–6× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.5% sodium dodecyl sulfate (SDS) at 65°C, hybridized at 55°C in the same solution, and washed in 3× SSC–0.5% SDS at 55°C (low stringency) or in 0.1× SSC–0.5% SDS at 68°C (high stringency).

Preparation and screening of the bacteriophage λ library. Genomic DNA prepared from *A. pleuropneumoniae* AP76 was partially digested with *Sau*3A1. Fragments of 10 to 20 kb were purified by sucrose density centrifugation (17) and ligated into the *Bam*HI site of the λ2001 vector. The DNA was packaged by using a commercially available λ packaging system as specified by the manufacturers. The bacteriophage recombinants were titrated on *E. coli* NM539, replica-plated onto nitrocellulose disks, and screened by plaque hybridization with the pAA210-derived *lktA* gene as a probe. Washes were done under low-stringency conditions. Positive plaques were purified, and bacteriophage DNA was prepared and analyzed by Southern blot hybridization.

Preparation of antisera. Mouse antibodies to *A. pleuropneumoniae* serotype 1 strain AP37 supernatant were raised by intraperitoneal injection of ethanol-precipitated culture supernatant in complete Freund's adjuvant. To raise serum against the recombinant cytolysin, we prepared aggregate protein from *E. coli* transformed with pCY76/503 (a construct which contains the *Bgl*II fragment of λCY76/5 cloned into the *Bgl*II site of pGH432; the resulting 70K protein consists of five vector-derived amino acids on its amino terminus fused to the 726 carboxy-terminal amino acids of the cytolysin [data not shown]). Then, 30 μg of aggregate protein solubilized in guanidine hydrochloride and mixed with complete Freund's adjuvant was injected intraperitoneally. In both cases the animals were boosted 2 weeks later by subcutaneous injection of the respective antigen in incomplete Freund's adjuvant.

Immunoblots. Bacterial colonies were replica plated onto nitrocellulose disks and lysed in chloroform vapor. Nonspecific binding was blocked by incubation with 0.4% gelatin in washing buffer (150 mM sodium chloride, 10 mM Tris-HCl [pH 8.0], 0.05% Tween 20). Mouse sera against *A. pleuropneumoniae* serotype 1 culture supernatant and goat anti-mouse alkaline phosphatase conjugate (Kirkegaard and Perry Laboratories, Inc., Gaithersburg, Md.), both in washing buffer, were subsequently added, and each was incubated for 1 hour at room temperature. The colony blots were developed with substrate containing Nitro Blue Tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate (BCIP). Protein gels were electroblotted onto nitrocellulose (31), blocked, reacted, and developed as described above.

Nucleotide sequence analysis. DNA sequencing was performed by using M13 vectors and the dideoxy-chain termination method essentially as described by Sanger et al. (22). Nested deletions were prepared by exonuclease III treatment, and specific primers were prepared by using a Pharmacia Gene Assembler. Sequences were analyzed by using the IBI/Pustell program and the GenBank network. Both strands of the *Bam*HI-*Bgl*II fragment of λCY76Δ1/1 were sequenced; one strand was sequenced for each of the two repeats in λCY76/5.

Nucleotide sequence accession number. The GenBank nucleotide sequence data base accession number for the repeat is M7488.

RESULTS

Cloning, expression, and characterization of the cytolysin A gene (*cytA*). Screening of the genomic library resulted in the identification of several hybridizing plaques. The initial mapping of these phages revealed that one clone, designated λCY76/5, had the *cytA* gene located in the center of a 17-kb insert (Fig. 1). Also, the hybridization pattern of the recombinant phage DNA digested with *Eco*RI, *Cla*I, *Bgl*II, *Xba*I, *Kpn*I, and *Hin*dIII matched that of the genomic DNA (data not shown). Subsequently, restricted DNA from this clone as well as from plasmid and genomic DNA preparations derived from *A. pleuropneumoniae* AP76 and AP205 was probed with the λCY76/5 insert DNA and with a probe containing parts of the *Pasteurella haemolytica lktB* and *lktD* genes. No hybridization was detected with the DNA obtained from λCY76/5, whereas the total genomic DNA from *A. pleuropneumoniae* AP76 hybridized strongly (Fig. 2a), thus indicating that the *cytA* gene is not clustered with the *lktB* and *lktD* analogous genes. Also, the plasmid preparations from *A. pleuropneumoniae* AP76 and AP205 did not hybridize or only weakly hybridized to the λCY76/5-derived probe, and the hybridizing fragments differed in size from those seen in the total genomic DNA preparations (Fig. 2b). This indicates that the *cytA* gene is not plasmid encoded. An initial nucleotide sequence analysis from both *Bgl*II sites toward the center of the clone established the direction of transcription of *cytA* as well as its reading frame by comparing it with the *A. pleuropneumoniae* hemolysin sequence previously determined by Chang et al. (2).

Isolation and characterization of spontaneous mutants. *A. pleuropneumoniae* AP76 and AP205 were subcultured twice from single colonies. Then two independent serial dilutions were made for each strain, and approximately 10,000 colonies were plated from each. After replicaplating onto nitrocellulose, three independent cytolysin-negative colonies were detected by immunoblot and designated AP76Δ1, AP205Δ1, and AP205Δ2. Western immunoblot analysis of whole-cell lysates revealed that these colonies lacked the cytolysin, whereas the Coomassie blue-stained total protein profile appeared to be identical with the wild type (Fig. 3). Southern blot analysis of restricted DNA from AP76Δ1 and AP205Δ1 with λCY76/5-derived probes revealed that the *Bgl*II fragment was absent, although hybridization was observed after the *Bgl*II fragment was used as a probe (Fig. 4a). Hybridization with the *Bgl*II-*Eco*RI fragments located on either end of λCY76/5 resulted in the appearance of strong bands in the cytolysin-negative mutants (Fig. 4b and c), and the hybridizing *Eco*RI fragment appeared to be approximately 7 kb smaller than that in the wild type. The hybrid-

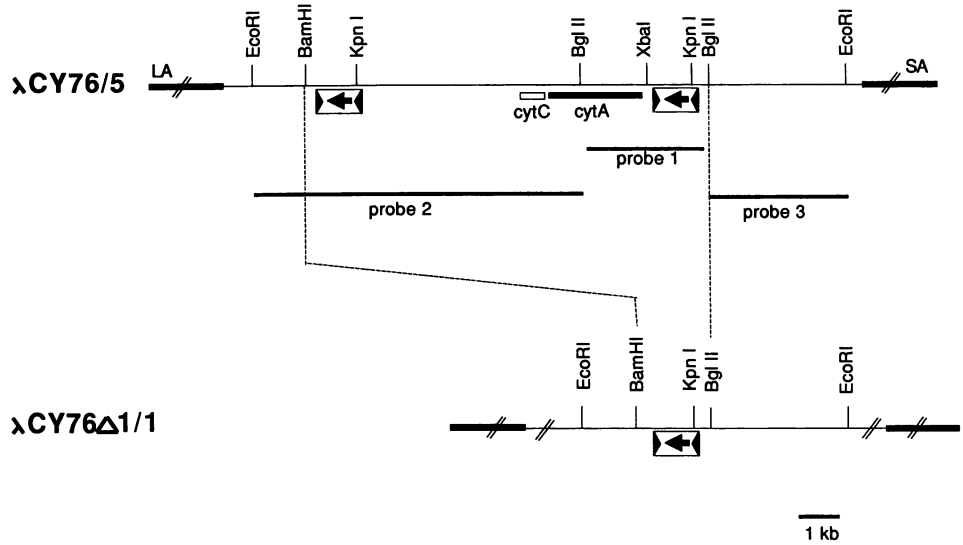

FIG. 1. Physical map of λCY76/5 and λCY76Δ1/1. The thick line represents DNA of the cloning vehicle (λ2001), with LA (long arm) and SA (short arm) giving the orientation of the insert DNA; the open *cytC*-labeled bar indicates the relative size and position of the *cytC* gene as determined by Chang et al. (2). The *cytA*-labeled bar indicates the position and relative size of the *cytA* gene as determined by expression and immunoblot (data not shown). The open boxes indicate the location and relative size of the direct repeats. The enclosed arrows show the direction of the open reading frame. The solid triangles on either end represent the inverted repeats. The thick lines underneath λCY76/5 designated as probes 1, 2, and 3 indicate the position of the probes used in Fig. 4. The dashed lines connect matching restriction enzyme sites on λCY76/5 and λCY76Δ1/1.

ization of multiple bands in Fig. 4a and b was subsequently explained by the detection of identical repeats (see below) (Fig. 1) and confirmed by using a probe which contained only the repeat sequence (data not shown).

Characterization of the *cytA* excision site. A genomic library was prepared from AP76Δ1 and probed with the *Eco*RI fragment derived from λCY76/5. Several clones were isolated, and initial characterization revealed that one clone had a *Bam*HI-*Kpn*I fragment identical in size to that of λCY76/5. This clone was designated as λCY76Δ1/1. Also, the nucleotide sequence of the *Bam*HI-*Kpn*I fragment of this clone (Fig. 5) was identical to the corresponding region of λCY76/5. Part of this sequence was present a second time on λCY76/5 starting 358 bp downstream from the end of *cytA* (Fig. 6). Further analysis showed that *cytA* is flanked by two identical direct repeats, each 1,201 bp in length, and that one repeat is completely conserved in λCY76Δ1/1. The sequence flanking the direct repeats located on either site of the *cytA* gene in λCY76/5 is TTAATG...AATATT. This sequence is identical to that flanking the single repeat in λCY76Δ1 (Fig.

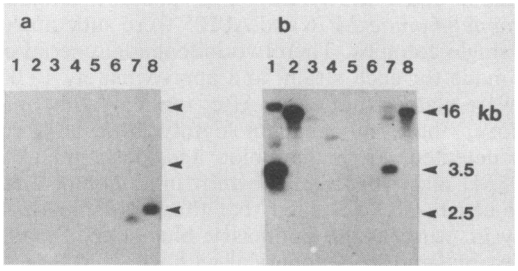

FIG. 2. Southern blot analysis with a pAA210-derived *lktB/D*-probe (a) and a probe composed of the *Eco*RI fragment of λCY76/5 (b). Both blots were washed under low-stringency conditions. The different lanes contain DNA from λCY76/5 (lanes 1 and 2), plasmid DNA from *A. pleuropneumoniae* AP76 (lanes 3 and 4) and AP205 (lanes 5 and 6), and chromosomal DNA from *A. pleuropneumoniae* AP76 (lanes 7 and 8). DNA in odd-numbered lanes is restricted with *Bgl*II, and DNA in even-numbered lanes is restricted with *Eco*RI. The arrowheads on the right indicate the position of size markers in kilobase pairs.

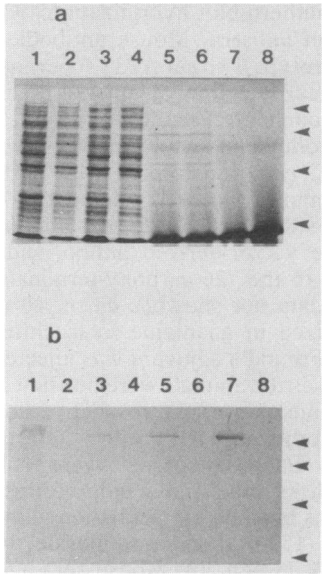

FIG. 3. Coomassie blue stain (a) and Western blot (b) of *A. pleuropneumoniae* wild type and cytolysin-negative mutants. The different lanes contain whole-cell lysates of *A. pleuropneumoniae* AP76 (lane 1), AP76Δ1 (lane 2), AP205 (lane 3), and AP205Δ1 (lane 4). Lanes 5 to 8 contain ethanol-precipitated supernatants from the same strains. The arrowheads on the right indicate the positions of size markers (phosphorylase *b*, 97,400; bovine serum albumin, 66,200; ovalbumin, 45,000; carbonic anhydrase, 31,000).

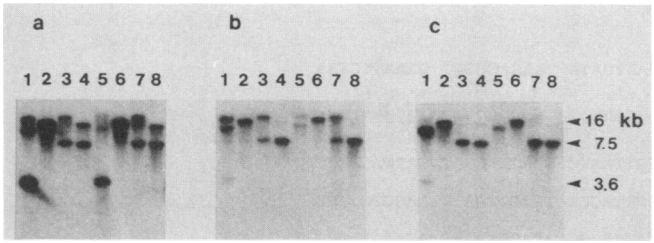

FIG. 4. Southern blot analysis of *A. pleuropneumoniae* wild type and cytolysin-negative mutants with probes 1 (a), 2 (b), and 3 (c) as indicated in Fig. 1. The blot in panel a was washed at low stringency; the other two blots were washed at high stringency. The different lanes contain DNA from *A. pleuropneumoniae* AP76 (lanes 1 and 2), AP76Δ1 (lanes 3 and 4), AP205 (lanes 5 and 6), and AP205Δ1 (lanes 7 and 8). DNA in odd-numbered lanes was cut with *Bgl*II, and DNA in even-numbered lanes was cut with *Eco*RI. The arrowheads on the right give the positions and lengths (in kilobases) of DNA size markers.

1), and it is not part of an apparent longer reading frame (Fig. 5). An initial analysis of the repeat sequence revealed that its ends form complementary repeats with four mismatches over a length of 26 bp. They also contain one open reading frame going in the opposite direction to *cytA*. The open reading frame is 1,038 nucleotides long and is preceded by a Shine-Dalgarno consensus sequence. Both the nucleotide sequence of the repeat and the predicted amino acid of the open reading frame were subjected to a homology search by using the GenBank data base, and no likely similarities (>65% on the nucleotide level or >35% on the amino acid level) were observed.

DISCUSSION

In the present communication we have characterized the organization of the region of the *A. pleuropneumoniae* chromosome flanking the *cytA* gene. We observed that the *cytA* gene is not clustered with other genes encoding various transport proteins, as has been described for homologous

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   1 GGATCCTGTT CTTGGTGAAA GTGTGGAACT TAAAGTTAAC TTATGTTTAG AGAAAAAAGG
     BamHI
  61 ATGGTATCTA GAGCAAGGTC CAGTGTGTGA AGAAAAATAC GTATGGAATG AACCGGAATG

 121 TATTAAATGG CGAGCAAAAT ATAGTAAGCC AAATGTGCAA CCTTGGGGAT AATAGTCATT

 181 TAAGTGTTTT AAAAATTTAA TTTCAGAAAT TTGTAATGGA TACAATGAAT ACAGAAAATA

 241 ATTAATGTTT AAAATCAAGC ACTAAATGAT TTTGTAATGG CACTTTAGCT GGGGTTATAT

 301 GAAGTAAATT CTTAATGTGT AGAAAATCAA ACCTAATCTG ACAGTTCCCG TTTAAAATTA
                    inverted repeat
 361 CCGTGTCTGT CAGATTAATT TGAGCTTAAA TTCTTTTCTG CCCAAATCCG TTTTCCATCA
               *** <------ end of open reading frame
 421 AGTAATGTTG CCATCGGTGT TCTGCCACAG CACACTTTTC CTTGATGTGT TCGATGGTGA

 481 TTATAATACA TTAACCACTC ATCTAAATCA GCTTGTAATG TCGCTAAATC CGTATATATT

 541 TTCTTCCTAA ATGCGACTTG GTAAAATTCT TGTAAGATAG TCTTATGAAA ACGTTCACAG

 601 ATACCATTCG TCTGTGGATG CTTCACTTTC GTTTTAGTAT GCTCTATGTC ATTTATCGCT

 661 AAATAAAGCT CATAATCGTG ATTTTCCACT TTGCCACAAT ATTCACTGCC ACGGTCGGTG

 721 AGAATACGCA ACATCGGTAA TCCTTGGGCT TCAAAGAACG GCAGTACTTT ATCATTGAGC

 781 ATATCTGCAG CGGCAATTGC GGTTTTCATT GTGTAGAGCT TTGCAAAAGC AACCTTACTA

 841 TAAGTATCAA CAAATGTTTG CTGATAAATG CGTCCAACAC CTTTTAAATT ACCTACATAA

 901 AAGGTATCTT GTGAACCTAA ATAGCCCGGA TGAGCGGTTT CAATTTCTCC ACTCGATATA

 961 TCATCCTCTT TCTTACGTTC TAGGGCTTGG ACTTGACTTT CATTTAGAAT AATGCCTTTC

1021 TCAGCCACTT CTTTCTCTAG TGCATTTAAA CGCTGTTTAA AGTTAGTAAG ATTATGACGT

1081 AGCCAAATGG AACGAACACC ACCGGCTGAA ACAAACACAC CTTGCTTGCG AAGTTCGTTA

1141 CTCACTCGAA CTTGTCCGTA AGCTGGAAAA TCTAGAGCAA ATTTTACAAC AGCTTGCTCA

1201 ATGTGCTCGT CTACTCGATT TTTGATATTC GGTACCCGAC GAGTTTGCTT AACTAATGCT
                                  KpnI
1261 TCAACACCGC CTTGCGCTAC GGCTTGTTGA TAGCGATAGA ATGTATCTCG GCTCATTCCC

1321 ATCGCTTTAC AAGCTTGAGA AATGTTTCCG AGTTCTTCTG CTAAATTGAG TAAACCGGTC

1381 TTGTGTTTAA TGAGCGGATT GTTAGAATAA AACATGAGAG TTTCCTTTTT TGTTTAGATT
       start of open reading frame <---- MET      SD
1441 GAATTTTAGA CACTCATATT CTAAACGGGA AACTCTCATT TTTATAATGA TTTGTCAGAT

1501 CAAGTCTGAT CTTCTACAAA TATTATCCCC ATTTATGGAG TTCGTCTTTT AGATGAACTC
     inverted repeat
1561 CTATTGTTTA TAATTCGATA AAATTAGCTT TCTCACAGCA ACTCAGCAAT GGGTTGCTTT

1621 TTTATTTGAC AGAAAAACAA CGTAGATCT
                           BglII
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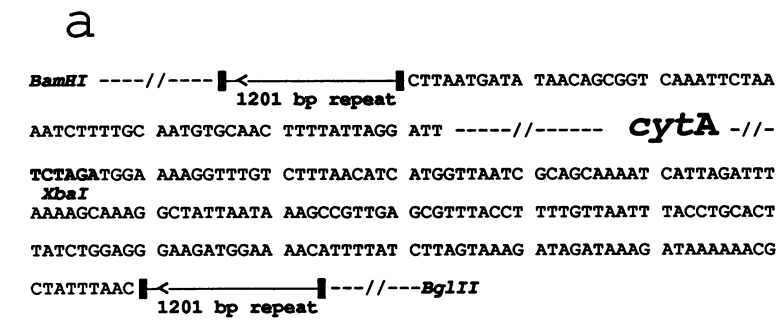
FIG. 5. Nucleotide sequence of the *Bam*HI-*Bgl*II fragment of λCY76Δ1/1. *Bam*HI, *Kpn*I, and *Bgl*II indicate the positions of the restriction enzyme sites also marked in Fig. 1. The position and direction of the open reading frame are indicated by *MET* and ***. *SD* marks the Shine-Dalgarno consensus sequence. The ends of the repeat are composed of inverted 26-bp inverted repeats, emphasized by boldface type.



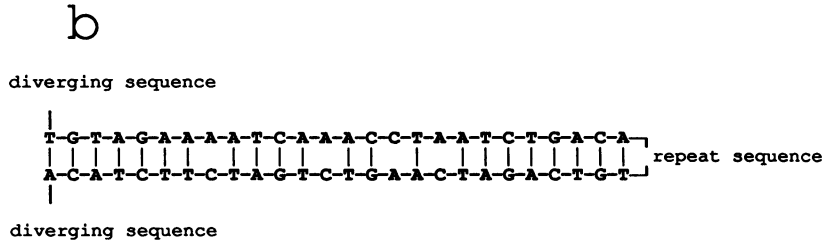


FIG. 6. (a) Nucleotide sequence of the flanking regions of the repeats on λCY76/5. The *Bam*HI and *Bgl*II sites are those connected to the respective sites on λCY76Δ1/1 in Fig. 1; *cyt*A marks the position of the *cytA* gene, and the sequence at the *Xba*I site and upstream is identical to that described by Chang et al. (2). (b) Nucleotide sequence of the inverted repeats located on either end of the direct repeats. Complementary bases are connected with a vertical dash.

genes in other organisms. Thus, in *E. coli* the *cytA* gene analog is located in an operon together with two genes (*hlyB* and *hlyD*) necessary for membrane transport (13, 35). In *P. haemolytica* and in *A. pleuropneumoniae* serotype 1 the *cytA* gene analog is clustered with two genes necessary for membrane transport (6, 30), and in *Bordetella pertussis* a third gene encoding a transport function is located immediately downstream from a *hlyD* analog (7). On the other hand, the molecular organization of another family of pore-forming toxins commonly found in *Proteus mirabilis* and *Serratia marcescens* (14, 21) shows no evidence for the presence of B and D genes downstream from the A gene, and a protein encoded upstream from the A gene is responsible for secretion of these toxins (21, 32). Our hybridization data show strong homology with the *lktA* gene and the presence of *lktB*- and *lktD*-analogous genes elsewhere on the *A. pleuropneumoniae* chromosome. These results indicate that the ±103K protein is a member of the RTX toxin family. This conclusion is supported by the results of our nucleotide sequencing analysis, showing that the sequence downstream from the *Bgl*II site located within the *cytA* gene is identical to that obtained by Chang and et al. for the *A. pleuropneumoniae* serotype 5 cytolysin (2). This group also showed the presence of an open reading frame upstream of the *cytA* gene which has a high degree of similarity with the *hlyC*/*lktC* genes of *E. coli* and *Pasteurella haemolytica* and not with the transport-associated gene found upstream of the *hlyA* gene in *P. mirabilis* and *S. marcescens*.

The ±103K cytolysin of *A. pleuropneumoniae* is widely distributed among different serotypes, whereas the ±105K and the ±110K cytolysins are found in only certain serotypes (12). Because a plasmid-encoded nature of the cytolysin would explain this discrepancy and because a hemolysin-encoding plasmid has been found in *E. coli* (19, 26), we examined plasmid preparations from two different *A. pleuropneumoniae* serotype 7 strains by Southern hybridization. The results indicated that the ±103K cytolysin, in this serotype, is not commonly plasmid encoded. However, it is possible that the cytolysin is plasmid encoded in other strains or serotypes. Thus, it was shown that a *Streptomyces* plasmid, depending on the host strain, was either maintained as a plasmid or integrated into the chromosome (9, 20).

In *E. coli* an IS*2* element has been found upstream of the hemolysin gene cluster (33). Also, the importance of other insertion sequences has been described for the transfer of the *E. coli* STI and STII toxins (10, 27), and it has been speculated that IS*1* might play a role in the integration of the *Shigella* virulence plasmid into the chromosome (3).

To see whether an insertion sequence could be involved in the widespread distribution of the ±103K cytolysin, we investigated whether we could isolate spontaneous noncytolytic mutants from two different *A. pleuropneumoniae* serotype 7 isolates. Our results indicate that a switch to a noncytolytic phenotype occurred with a high frequency, and further investigation showed that the phenotypic switch was associated with the loss of the encoding gene. The two direct repeats found at either end of the excised DNA fragment, as well as the one remaining copy of this repeat in the spontaneous mutant, strongly indicate that the loss of the *cytA* gene is due to a homologous recombination event. The location of the direct repeat also explains the hybridization of the *A. pleuropneumoniae* AP76Δ1 and AP205Δ1 DNA with the *Bgl*II fragment from λCY76/5 used as a probe. Thus, this DNA fragment contains one repeat region allowing hybrid-

ization to the repeat that is conserved after spontaneous deletion of *cytA*.

There is complete identity of the repeats flanking the *cytA* gene. Also, each of the repeats was flanked by nearly identical inverted repeats showing four mismatches over their length of 26 nucleotides. The repeats contain one open reading frame preceded by a Shine-Dalgarno consensus sequence, thus suggesting that translation does occur. Overall, the repeats have the size and structure of insertion sequences, and the whole organization of the *cytA* gene and its flanking regions strongly resembles that of known transposable elements, except that it contains directly repeated insertion sequence-like elements on its ends. Therefore, it could be imagined that an inversion of one of these elements would result in a functional transposable element. Alternatively, it has been shown that for the transposition of Tn*4521* (the transposon carrying the STII-encoding gene), an insertion sequence on only one end is required (10). This possibility of transposition would provide an interesting explanation for the frequent occurrence of the ±103K cytolysin among *A. pleuropneumoniae* serotypes. However, it remains to be investigated whether the sequence actually has transposition ability, whether the open reading frame is translated, and whether the mismatches in the terminal inverted repeats possibly influence transposition frequency.

ACKNOWLEDGMENTS

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ADDENDUM IN PROOF

The unlinked nature of the *A. pleuropneumoniae cytC* and *cytA* genes coding for cytolysin transport functions has recently been reported (Y.-F. Chang, R. Young, and D. K. Struck, J. Bacteriol. **173**:5151–5158, 1991).

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