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Molecular Cloning and Characterization of a Hemolysin Gene from *Actinobacillus (Haemophilus) pleuropneumoniae*

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This article describes the molecular cloning and expression of a hemolysin gene from a serotype 1 strain of *Actinobacillus pleuropneumoniae*. The hemolysin was a thermolabile protein with an apparent molecular weight of 29,500 (29.5K hemolysin). Unlike expression of the recently described 105K hemolysin of *A. pleuropneumoniae* (J. Frey and J. Nicolet, FEMS Microbiol. Lett. 55:41–46, 1988), expression of this hemolysin was not regulated by Ca^{2+}. Antiserum prepared against the 105K hemolysin did not neutralize the activity of the 29.5K hemolysin; conversely, antiserum prepared against the 29.5K hemolysin did not neutralize the activity of the 105K hemolysin. The hemolytic activity was not neutralized with antisera against hemolytic *Escherichia coli*, *Streptococcus agalactiae*, or purified streptolysin O, but antisera prepared against recombinants containing the 29.5K gene and convalescent pig sera abrogated hemolytic activity. Although hemolytic activity could be detected in several strains of *E. coli* K-12 and in minicells expressing several different constructs encoding the 29.5K hemolysin, we could not rigorously exclude the possibility that the gene which we have isolated encodes a regulator of hemolytic activity rather than a hemolysin per se.

Actinobacillus (Haemophilus) pleuropneumoniae is an important pathogen of swine throughout the world (19, 26). The organism can cause an acute and frequently fatal pleuropneumonia which is marked by extensive hemorrhagic lesions and fibrin exudation in the lungs (2, 19, 22). Similar lesions can be induced by using bacterium-free culture supernatants, suggesting that *A. pleuropneumoniae* can elaborate a toxin or toxins (24).

Hemolytic activity, often associated with neutrophil cytotoxic activity, has been described for the majority of serotypes of *A. pleuropneumoniae* (23). There is growing evidence, however, that there may be several distinct hemolysins associated with different strains of *A. pleuropneumoniae*. Kume et al. reported a heat-stable hemolytic activity in serotype 2 strains mediated by a carbohydrate molecule (12, 17). An RNA-dependent hemolytic activity from *A. pleuropneumoniae* in serotype 1, 2, and 5 strains has been described by Martin et al. (15). Most recently, Frey and Nicolet (7) and Devenish and Rosendal (4) have reported the protein purification of a potent 105,000-molecular-weight hemolysin (105K hemolysin) from serotype 1 strains. The expression of this hemolysin is strongly dependent on the presence of free Ca^{2+} (8). Frey and Nicolet have also described Ca^{2+}-independent hemolytic activity associated with serotypes 2, 4, 7, and 8 (J. Frey and J. Nicolet, Conf. Res. Work. Anim. Dis., abstr. no. 370, p. 66, 1988).

In order to better understand the role of *A. pleuropneumoniae* hemolysins in virulence, we prepared a gene library of a highly virulent serotype 1 strain, CM5. A strongly hemolytic recombinant was isolated, and the physicochemical properties of that hemolytic activity were determined. The recombinant hemolysin was clearly different from the 105K hemolysin previously described.

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MATERIALS AND METHODS

Bacterial strains and cultivation. CM5, a serotype 1 strain of *A. pleuropneumoniae* isolated from an acute case of pleuropneumonia, was used for the construction of the gene library. The sources of the *Actinobacillus*, *Haemophilus*, and *Pasteurella* spp. strains used in the Southern blotting experiments have been described previously (13) except for *A. pleuropneumoniae* serotype 5b strain L20, serotype 9 strain CVJ 13261, serotype 10 strain D13039, serotype 11 strain 56513, and serotype 12 strain 8329. These strains were the generous gift of R. Nielsen, Copenhagen, Denmark. In addition, SQ4 (a hemolytic *Actinobacillus suis* field strain), 41840 (a *Pasteurella multocida* field strain), 43826 (a *Haemophilus somnus* field strain), and 4429 and P155 (hemolytic *E. coli* strains) were included. The *Haemophilus*, *Actinobacillus*, and *Pasteurella* spp. isolates were grown at 37°C on chocolate blood agar containing 0.01% NAD (wt/vol) (13).

E. coli K-12 strains TB1 and frozen competent DH5α (Bethesda Research Laboratories Life Technologies, Inc., Gaithersburg, Md.) were used as recipients for the propagation of recombinant plasmids. *E. coli* χ^{984} was used for the production of minicells.

Cloning of the hemolysin gene. The cloning and subcloning procedures used in this study were essentially as described by Maniatis et al. (14). Total bacterial DNA was isolated by a modification of the method of Marmur (10, 27). Unless indicated otherwise, enzymes and cloning reagents were purchased from Pharmacia (Canada) Ltd., Baie d'Urfe, Quebec, Canada.

DNA was extracted from strain CM5 and partially digested with *Sau*3A. The *Sau*3A digest was fractionated by sedimentation through a sucrose gradient. Fragments of approximately 5 to 10 kilobase pairs (kbp) were ligated to *Bam*HI-digested pBR322 which had been treated with bacterial alkaline phosphatase (BAP) to prevent recircularization. After CaCl transformation into competent TB1 cells, transformants were selected on Luria-Bertani plates containing 50 μg of ampicillin per ml (14). Colonies which were ampicillin resistant and tetracycline sensitive were transferred onto blood agar (with 50 μg of ampicillin per ml) and

screened for hemolysis. One strongly hemolytic recombinant, A44, was selected for subcloning and further analysis.

Subcloning of the hemolysin gene. After restriction mapping with *Bam*HI, *Eco*RI, *Hin*dIII, and *Pst*I, recombinant A44 was digested with *Hin*dIII and ligated into *Hin*dIII-digested, BAP-treated pUC18. TB1 cells were transformed and hemolytic recombinants were selected as described above. All recombinants that contained the 6.1-kbp *Hin*dIII fragment were hemolytic. One of these, which we designated A44-4, was subcloned further. A44-4 DNA was digested completely with *Hin*dIII and then partially with *Eco*RI. The *Hin*dIII-*Eco*RI partial digest was ligated into *Hin*dIII-*Eco*RI-digested, BAP-treated pUC18 and transformed into competent DH5α cells. Hemolytic recombinants were selected as described above.

Generation of deletion clones. A44-4 DNA was digested with *Hin*dIII, and the resultant fragments were separated by agarose gel electrophoresis. The 6.1-kbp *Hin*dIII fragment was purified and ligated with *Hin*dIII digested, BAP-treated pGEM-4 (Promega Biotec, Madison, Wis.). Hemolytic recombinants with the *Hin*dIII fragment inserted in both orientations were isolated. The *Hin*dIII fragment was deleted from the 3′ and 5′ ends by using the Erase-a-Base system according to the instructions of the manufacturer (Promega Biotec). The restriction enzymes *Sac*I and *Sal*I were used to create the protected 3′ and the accessible 5′ ends, respectively. After ligation, deletion clones were transformed into competent DH5α cells. Hemolytic and nonhemolytic deletion clones were selected from various time points. The sizes of the deletions were determined by agarose gel electrophoresis of *Eco*RI-digested recombinant plasmids with *Hin*dIII-digested lambda DNA as a marker. The approximate sizes of the small DNA fragments were determined by polyacrylamide gel electrophoresis (27).

Southern blotting. DNAs were digested to completion with *Eco*RI, separated in a 0.7% agarose gel, and transferred to BioTrans nylon membranes (ICN Pharmaceuticals Inc., Irvine, Calif.). A 480-bp *Eco*RI fragment from deletion clone 2-12 was gel purified and labeled with [^{32}P]dATP by random priming according to the instructions of the manufacturer (Boehringer Mannheim, Canada, Dorval, Quebec, Canada). The transfer, hybridization, and washing of the Southern blots were performed essentially as described elsewhere (14). Bacteriophage lambda DNA (strain *c*I857 *S*am7), cut with *Eco*RI and *Hin*dIII, was used as a molecular weight marker. Digestion of the lambda DNA produced fragments of 21,226, 5,148, 4,973, 3,530, 2,027, 1,904, 1,584, 1,330, 983, 831, 564, and 125 bp.

Characterization of the physicochemical properties of the recombinant hemolysin. The procedures for determining Ca^{2+} requirements, heat, cholesterol and protease sensitivity, neutrophil cytotoxicity, hemolytic activity, and neutralization have been described elsewhere (8, 23). Unless indicated otherwise, neutralization experiments were done with a 1:100 dilution of antisera. Appropriate preimmune antisera of the same species at the same dilution were used as controls for nonspecific inhibition of hemolytic activity.

Antisera. Antisera against the 105K hemolysin were prepared as described previously (4). Rabbit antiserum was also prepared against ammonium sulfate-precipitated, sonicated A44 and A44RI (13, 23). Rabbit antiserum prepared against a hemolytic *E. coli* strain was supplied by C. Gyles (University of Guelph, Guelph, Ontario, Canada). Horse antiserum against streptolysin O was provided by J. Alouf (Institut Pasteur, Paris, France).

In vitro expression. A44 subclones and deletion clones were expressed in the *E. coli* minicell-producing strain X^{984} as described except that Luria-Bertani broth was used in the place of BSG buffer and the cycloserine step was omitted (21). The preparations were contaminated with less than one viable bacterium per 10^6 minicells. Deletion clone T44 was expressed in an in vitro transcription-translation system according to the instructions of the manufacturer (Amersham Canada Ltd., Oakville, Ontario, Canada). On the basis of DNA sequence information (J. MacInnes and C.-J. Lian, manuscript in preparation), [^{35}S]methionine (Amersham) was used for labeling.

Preparation of protein extracts and immunoblotting. Periplasmic proteins were extracted from recombinants and *A. pleuropneumoniae* CM5 by using osmotic lysis of polymyxin B-treated cells (5). Recombinant, *A. pleuropneumoniae*, and periplasmic proteins were immunoblotted by a modification of the method of Towbin (13). Antiserum against A44 hemolysin and semipurified 105K hemolysin was absorbed twice with *E. coli* TB1 cells (13).

RESULTS

Genetic analysis of hemolytic recombinants. A gene library of the highly virulent *A. pleuropneumoniae* CM5 was prepared in the plasmid vector pBR322. Although only approximately 900 ampicillin-resistant, tetracycline-sensitive transformants were recovered, 25 showed some degree of hemolytic activity on blood agar plates. The sizes of the inserts in these hemolytic recombinants ranged from 3.5 to 13 kbp. On the basis of limited restriction mapping, few of these recombinants appeared to contain common sequences. The most strongly hemolytic recombinant, which we designated A44, was selected for further analysis.

Recombinant A44 contained a 12.4-kb insert (Fig. 1). Subcloning experiments revealed that the hemolytic activity was associated with the 6,100-bp *Hin*dIII fragment. It was further shown that the hemolytic activity was encoded by a 2,100-bp *Hin*dIII-*Eco*RI partial fragment which included approximately 350 bp of pBR322 sequence.

To determine the size of the hemolysin gene more precisely and to ascertain whether we had cloned a complete hemolysin gene or a gene which was fused to the tetracycline promoter of pBR322, we prepared a series of deletion mutants. The 6.1-kbp fragment was inserted in pGEM-4 in both orientations and deleted from the 3′ and 5′ ends. The sizes and the phenotypes of representative deletion clones are listed in Table 1. Several 5′-end deletion clones from which all pBR322 sequences had been eliminated were hemolytic. In fact, all 5′-end deletions of less than 525 bp did not affect hemolytic activity. All 3′-end deletions which contained at least 1,250 bp were also hemolytic.

Physicochemical properties of the recombinant hemolysin. In order to compare the A44 hemolysin with the 105K hemolysin of *A. pleuropneumoniae* (8, 9, 23) and other established classes of hemolysins (1, 3, 11, 20, 28, 30), several physicochemical properties of the recombinant hemolysin were determined (Table 2). Both hemolysins were inactive after heat or protease treatment. Like 105K-hemolysin activity, A44 hemolytic activity was inhibited in the presence of cholesterol. The two hemolytic activities were Ca^{2+} independent, but unlike 105K-hemolysin expression, expression of the A44 hemolysin was Ca^{2+} independent.

Antiserum prepared against semipurified A44 or A44RI hemolysin did not neutralize the hemolytic activity of a semipurified preparation of the 105K hemolysin. Conversely, antiserum against the semipurified 105K hemolysin

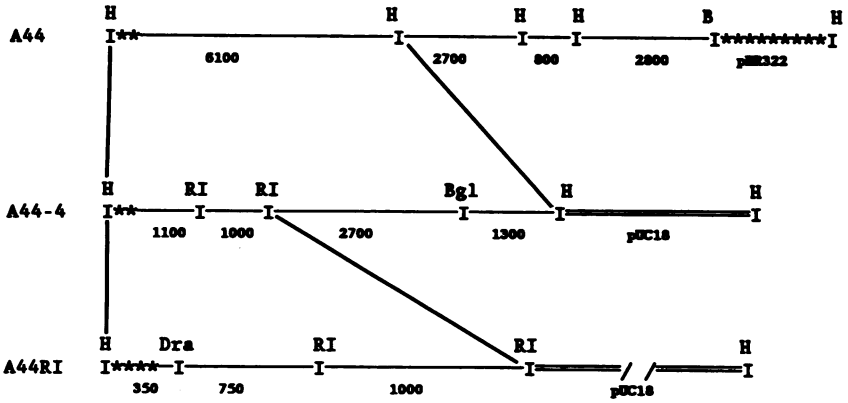

FIG. 1. Cloning strategy and restriction endonuclease maps of hemolytic recombinants. The relative positions of the 2,700- and 800-bp *Hin*dIII fragments of A44 were not determined unequivocally. The approximate sizes of the restriction fragments are indicated in base pairs. Restriction endonuclease sites: H, *Hin*dIII; B, *Bam*HI; RI, *Eco*RI; Bgl, *Bgl*II; Dra, *Dra*I.

failed to neutralize the hemolytic activity of semipurified A44 hemolysin. Convalescent serum from pigs infected with serotype 1 *A. pleuropneumoniae* was able to neutralize the 105K-hemolysin activity at a 1:100 dilution; A44 hemolytic activity was neutralized by two sera at 1:25 dilution and by another at a 1:10 dilution.

Antisera against several other hemolysins failed to neutralize the A44 hemolytic activity. Three convalescent antisera from cows infected with *Streptococcus agalactiae* failed to neutralize hemolytic activity. Similarly, neither antiserum prepared against hemolytic *E. coli* nor streptolysin O antiserum was able to neutralize hemolytic activity.

Southern blotting. To determine whether the A44 hemolysin was present in all serotypes of *A. pleuropneumoniae*, DNAs from the reference strains of serotypes 1 to 12 were examined by Southern blotting (Fig. 2, lanes 1 to 6 [serotypes 1 to 4, 5a, and 5b] and lanes 8 to 14 [serotypes 6 to 12]). A 480-bp *Eco*RI fragment from deletion clone 2-12 was used to probe *Eco*RI-digested total DNA. *Dra*I-*Eco*RI-digested A44 DNA (Fig. 2, lane 7) and *Eco*RI-digested CM5 DNA (lane 15) were included as controls. In all cases, a 3.3-kbp fragment was detected. Several additional bands (4.0, 16.6, and 21.7 kbp) were also seen in serotype 3 DNA (Fig. 2, lane 3).

Under stringent conditions, sequences with homology to the A44 hemolysin sequence were also detected in serotype 1 field strain WLN (Fig. 3, lane 1) and in *Actinobacillus lignieresii* (lane 3). A larger, 6.8-kbp fragment was detected in *A. suis* (Fig. 3, lane 2). No homology could be detected with DNAs from *Haemophilus parasuis* (Fig. 3, lane 4), *Haemophilus influenzae* (lane 5), *Pasteurella haemolytica* (lane 7), *P. multocida* (lane 8), *H. somnus* (lane 9), *Haemophilus* minor group strains PN33 and 202 (lanes 10 and 11), hemolytic *E. coli* strains (lanes 12 and 13), *E. coli* DH5α (lane 14), or *Hin*dIII-*Eco*RI-digested lambda DNA (lane 15).

In vitro expression of the recombinant hemolysin. The deletion recombinant T44, which contained the hemolysin gene plus approximately 540 bp upstream, was transformed into a minicell-producing strain of *E. coli*, χ^{984}. As controls, pBR322 and pUC18 were also transformed. The plasmid-encoded proteins expressed in the minicells were labeled with [^{35}S]methionine, separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and visualized by autoradiography (Fig. 4A). Two unique bands of 29.5K and 16.5K were detected in the minicells carrying the T44 plasmid (Fig. 4A, lane 3). Minor bands of approximately 27K and 18.5K were also detected, but comparable bands were sometimes present in the pBR322 and pUC controls (Fig.

TABLE 1. Endpoints and phenotypes of deletion clones[a]

Recombinant	Position of:		Hemolysis
	First base (5′ end)	Last base (3′ end)	
2-36	400	6100	+
2-12	480	6100	+
2-71	525	6100	+
3-1	670	6100	−
4-7	730	6100	−
T26	1	890	−
D50	1	1025	−
T25	1	1125	−
T44	1	1250	+
T51	1	1530	+

[a] The construction of the deletion clones is described in the text. The positions of the 3′and 5′ deletions are indicated with respect to the pBR322 *Hin*dIII site. The first 346 bases of the recombinants contain bases 29 through 375 of pBR322.

TABLE 2. Physicochemical properties of the 105K and A44 hemolysins of *A. pleuropneumoniae*

Property	Result for hemolysin	
	105K	A44
Heat sensitivity[a]	+	+
Trypsin sensitivity[a]	+	+
Cholesterol sensitivity[a]	+	−
Ca^{2+} requirement for transcription[b]	+	−
Ca^{2+} requirement for hemolytic activity[b]	−	−
Neutralization by antibody to A44 hemolysin	−	+
Neutralization by antibody to 105K hemolysin	+	−
Neutralization by convalescent pig antiserum	+	+[c]
Apparent molecular weight	105K	29.5K

[a] Heat, trypsin, and cholesterol sensitivity determinations were made as described before (23). Briefly, the hemolysin was considered heat sensitive if all activity was abrogated by incubation at 60°C for 30 min, trypsin sensitive if all activity was abrogated by 0.05 mg of trypsin per ml at 37°C for 10 min, and cholesterol sensitive if all activity was abrogated in the presence of 1 mg of water-soluble cholesterol per ml at 37°C for 1 h.

[b] Hemolysins were deemed Ca^{2+} independent if they required less than 0.05 mM free Ca^{2+} (8).

[c] Two sera diluted 1:25 and one serum diluted 1:10 were neutralizing.

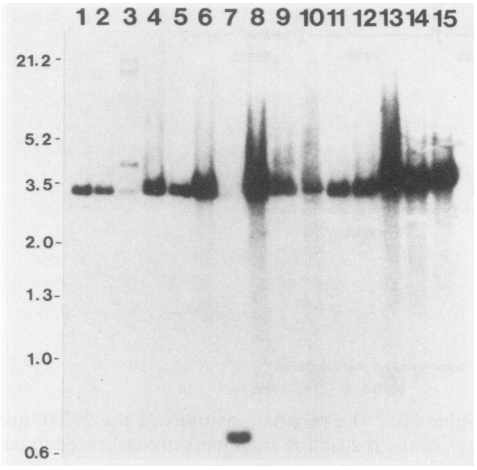

FIG. 2. Southern blot of total DNA of *A. pleuropneumoniae* serotypes 1 to 12 reference strains digested with *Eco*RI: Shope 4074 (lane 1), S1536 (lane 2), S1421 (lane 3), M62 (lane 4), K17 (lane 5), L20 (lane 6), *Dra*I-*Eco*RI A44 580-bp fragment (lane 7), Femϕ (lane 8), WF83 (lane 9), 405 (lane 10), CVJ 13261 (lane 11), D13039 (lane 12), 56513 (lane 13), 8329 (lane 14), and strain CM5 (lane 15). The blot was probed with the 480-bp *Eco*RI fragment of deletion clone 2-12. The positions of the molecular mass markers (lane 15) are shown on the left in kilobase pairs.

4A, lanes 1 and 2). Hemolytic activity was detected only in the minicells containing the T44 recombinant.

The T44 deletion clone was also expressed in an in vitro transcription translation system. Plasmids pAT153 and pGEM-4 were included as controls (Fig. 4B, lanes 1 and 2). In addition to the unprocessed β-lactamase gene product, a single major protein with an apparent molecular weight of 29,500 was encoded by T44 (Fig. 4B, lane 3). No hemolytic activity could be detected in the in vitro transcription-translation system.

Immunoblotting. When periplasmic protein extracts of DH5α cells containing A44, A44-4, and A44-RI were probed

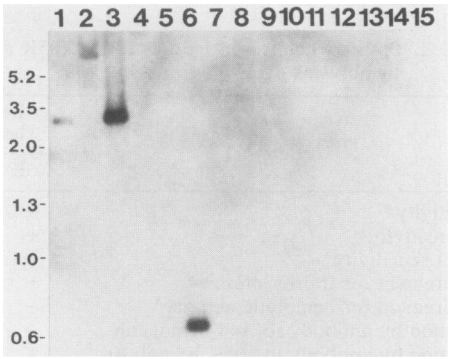

FIG. 3. Southern blot of total *Eco*RI-digested DNA of serotype 1 *A. pleuropneumoniae* field strain WLN (lane 1), *A. suis* SQ4 (lane 2), *A. lignieresii* ATCC 19393 (lane 3), *H. parasuis* ATCC 19417 (lane 4), *H. influenzae* ATCC 19418 (lane 5), *Dra*I-*Eco*RI-digested A44 (lane 6), *P. haemolytica* (lane 7), *P. multocida* (lane 8), *H. somnus* (lane 9), minor group strains PN33 (lane 10) and 202 (lane 11), hemolytic *E. coli* 4429 (lane 12) and P155 (lane 13), and *E. coli* DH5α (lane 14). The blot was probed with a 480-bp *Eco*RI fragment from A44-4. The sources of strains and the details of the procedures are given in Materials and Methods. The positions of the molecular mass markers (lane 15) are shown on the left in kilobase pairs.

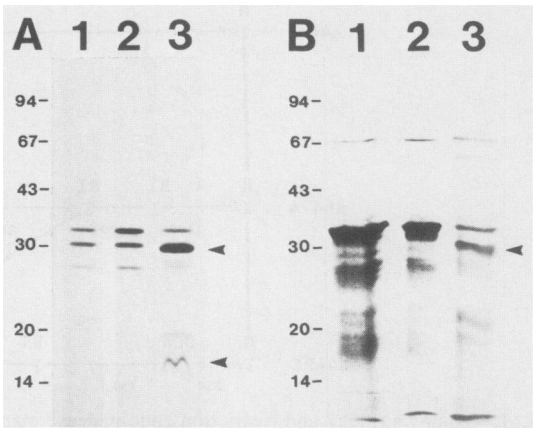

FIG. 4. In vitro expression of the A44 hemolysin. (A) [^{35}S]methionine-labeled plasmid-specific proteins encoded by pBR322 (lane 1), pUC18 (lane 2), and hemolytic recombinant T44 (lane 3). The positions of the 29.5K and 16.5K proteins are indicated by arrowheads. (B) [^{35}S]methionine-labeled proteins of in vitro transcription-translation of pAT153 (lane 1), pGEM-4 (lane 2), and T44 (lane 3). The position of the 29.5K protein is indicated by the arrowhead. The sizes of the molecular mass markers are indicated in kilodaltons.

with A44 antiserum, multiple proteins, including a 29.5K and a 14K protein, were detected (Fig. 5, lanes 3 through 5). No comparable bands were seen in the pBR322 and pUC control lanes (Fig. 5, lanes 1 and 2). The 14K protein was also detected in extracts of CM5 (Fig. 5, lane 6). There was no evidence of a 29.5K protein in CM5 extracts, although a major protein of approximately 27.5K was present.

DISCUSSION

Several virulence factors have been reported to contribute to the pathogenesis of *A. pleuropneumoniae* infection. An

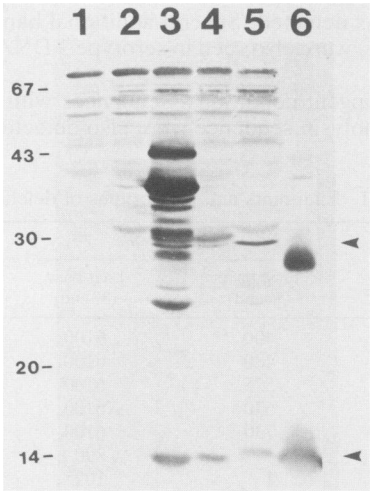

FIG. 5. Immunoblots of polymyxin B extracts of recombinants pBR322 (lane 1), pUC18 (lane 2), A44 (lane 3), A44-4 (lane 4), and A44RI (lane 5) and *A. pleuropneumoniae* CM5 (lane 6). The blots were probed with A44 antiserum absorbed with *E. coli*. The positions of the 29.5K and 14K proteins are indicated by arrowheads. The sizes of the molecular mass markers are indicated in kilodaltons.

intact capsule is required for virulence (9), and lipopolysaccharide endotoxin can mediate damage (6, 16, 29). However, in view of the very rapid clinical course of acute pleuropneumonia and the extensive vascular changes seen, it appears that one or more toxins play a major role in the disease process (2, 18, 22, 24).

Several hemolytic and cytolytic activities have been reported for different serotypes of *A. pleuropneumoniae* (4, 7, 8, 15, 17, 18). A 105K protein hemolysin has recently been purified and characterized (4, 7, 8). In the presence of free Ca^{2+}, this hemolysin is expressed at high levels in vitro. The 105K hemolysin is thought to be related to the α-hemolysin of *E. coli* (7). In this paper, we describe the cloning and characterization of a second protein hemolysin.

A gene library of the highly virulent serotype 1 strain of *A. pleuropneumoniae* was constructed in pBR322. Twenty-five hemolytic recombinants were obtained, including one strongly hemolytic clone, A44. The weakly hemolytic clones may represent truncated hemolysin genes or additional, less-active hemolysins.

By Southern blotting, we determined that sequences homologous to that of the A44 hemolysin were present in DNAs of serotypes 1 to 12 of *A. pleuropneumoniae*. A 3.3-kbp band could be detected in DNAs from all reference strains and several field strains (data not shown). Several additional bands were detected in DNA from serotype 3. The sizes of those fragments were consistent with partial digestion, but multiple bands were detected with three independent preparations of serotype 3 DNA and with four different enzymes. Either this DNA is especially difficult to digest or there are multiple copies of homologous sequences in *A. pleuropneumoniae* S1421.

In addition, homologous sequences were detected in *A. suis* and *A. lignieresii* but not in any of the *Haemophilus* strains examined (Fig. 3). Since all serotypes of *A. pleuropneumoniae* are pathogenic and only some serotypes possess the Ca^{2+}-regulated hemolytic activity, these data suggest that the A44 hemolysin may play a role in virulence.

The size of the active hemolysin has yet to be determined. When the deletion clone, T44, was expressed in minicells, two major plasmid-encoded polypeptides with apparent molecular weights of 29,500 and 16,500 were detected (Fig. 4A), whereas in an in vitro transcription-translation assay, only a 29.5K protein was observed (Fig. 4B). A 29.5K protein was also detected in immunoblots of polymyxin B extracts of recombinants. In CM5 extracts, a 14K protein was detected but there was no 29.5K protein present in significant quantities. Instead, the A44 antisera detected a 27.5K protein in CM5 extracts.

These data suggest that the primary A44 gene product is a 29.5K protein and that the protein is processed, or subject to breakdown, when expressed in *E. coli*. We do not know why the T44 construct appears to yield a larger cleavage product than do A44, A44-4, and A44RI constructs, but this yield may be related to the absence of specific downstream sequences. The protein also appears to be processed or broken down or both when expressed in *A. pleuropneumoniae* CM5. It is possible that strain CM5 processes the A44 hemolysin very efficiently (hence the 27.5K protein is abundant), whereas *E. coli* does not (hence the 29.5K protein is abundant). From DNA sequencing we know that the A44 gene encodes a protein with an apparent molecular weight of 29,500.

Even in the absence of processing, the A44 hemolysin is considerably smaller than the 105K hemolysin described earlier. The findings that expression of the A44 hemolysin is not Ca^{2+} regulated and that there is no antigen cross-reactivity between the two also argue that the A44 hemolysin activity is a separate and distinct hemolytic activity in *A. pleuropneumoniae*. The activity of the 29.5K hemolysin could be neutralized with antisera from *A. pleuropneumoniae*-infected pigs, but the titer was considerably lower than that to the 105K hemolysin. This result suggest that the 29.5K hemolysin may not be expressed at very high levels in vivo or that it is not strongly antigenic in swine.

Neutralization studies were performed to determine whether the A44 hemolysin was related to other established classes of hemolysins. The A44 hemolysin did not appear to share homology with *E. coli* hemolysin or with streptolysin O, and despite the fact that the 29.5K hemolysin is similar in size to protein B of *S. agalactiae* (25), no neutralization could be demonstrated with three independent convalescent sera from *S. agalactiae*-infected animals.

The fact that the minicells containing the A44 hemolysin were hemolytic suggests that the A44 hemolysin gene does encode a protein with hemolytic activity. Although hemolytic activity was not detected in the in vitro transcription-translation assay, this may be because extremely small amounts of protein were produced, because a cofactor was missing, or because processing is required to obtain an active form.

We cannot at this point rigorously exclude the possibility that A44 induces a hemolysin gene, as yet uncharacterized and with a very stable mRNA, found in a variety of *E. coli* K-12 strains or that the A44 gene product in some way activates a second protein. On the basis of DNA sequencing, A44 has no homology with known hemolysins or toxins but does share significant homology with a regulatory protein from *E. coli*, FNR (MacInnes and Lian, in preparation).

At least 40 hemolysins and cytolysins have been described to date (28). Some of these lysins, for example, the *E. coli* α-hemolysin family, the CAMP factor of *Streptococcus* spp., and the thiol-activated hemolysins of gram-positive organisms, have been well characterized (1, 3, 11, 28, 30). On the basis of its size and physicochemical properties, the A44 recombinant hemolysin described in this paper appears to be unique. Experiments are currently under way to determine its role in the pathogenesis of *A. pleuropneumoniae* infection.

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