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Hemolysin Patterns of *Actinobacillus pleuropneumoniae*

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The secreted hemolytic activities produced by the reference strains and field isolates of the 12 serotypes and 2 subtypes of *Actinobacillus pleuropneumoniae* were analyzed. Serotype 1 (reference strain 4074) produced a Ca^{2+}-inducible hemolysin, which was previously characterized as a 105-kilodalton protein and was named hemolysin I (HlyI). Serotypes 2, 4, 6, 7, and 8 produced a different hemolytic activity that was not inducible by Ca^{2+} but required this ion for its activity. The hemolytic activity produced by these serotypes was much weaker than that found in serotype 1 and was not neutralized by rabbit antibodies against HlyI. It was, however, neutralized by serum from pigs that were experimentally infected with a serotype 2 strain and was called hemolysin II (HlyII). Serotypes 5a, 5b, 9, 10, and 11 produced both HlyI and HlyII. In these strains, HlyI was the major contributor to the hemolytic activity. The remaining serotypes, 3 and 12, produced a very weak hemolytic activity, which was not further analyzed. Immunoblot analysis of the culture supernatants from all 12 serotypes with rabbit polyclonal antibodies directed against HlyI revealed reactions with a protein in the 105-kilodalton size range for all serotypes, indicating that HlyI and HlyII might be serologically related. Strains producing active HlyI seem to belong to serotypes that are generally considered to be virulent types and that are frequently isolated from pigs in severe pleuropneumonia outbreaks.

Actinobacillus pleuropneumoniae is the etiologic agent of pleuropneumonia, a contagious and often fatal respiratory disease of swine (32). In view of its importance in industrial pig production, this disease has become the subject of an intensive research effort. Twelve serotypes of *A. pleuropneumoniae* have been described on the basis of the serological typing of capsular polysaccharides (14, 22, 23, 25, 26, 30). In addition, serotype 5 was divided into subtypes 5a and 5b (24). The status of serotype 11 is unclear at the moment (25), since it cross-reacts strongly with serotype 9 (11). Similar serological cross-reactivity has also been demonstrated between serotypes 1 and 9, between serotypes 4 and 7, and among serotypes 3, 6, and 8 (21). In spite of extensive bacteriological and epidemiological research, the pathogenesis of infection still remains unclear. It seems likely that many virulence factors are involved, among them capsular polysaccharides (9), lipopolysaccharides (34), and hemolysins (7, 15, 19).

The hemolytic activity of *A. pleuropneumoniae* is characteristic of this species. The intensity of the hemolysis varies not only according to the type of erythrocyte used but also with the different serotypes (13; J. Nicolet, Ph.D. thesis, University of Berne, Bern, Switzerland, 1970). The hemolysin of serotype 1 strain 4074, hemolysin type I (HlyI), has been purified to homogeneity and characterized as a protein of 105 kilodaltons (kDa) which has some similarities with the *Escherichia coli* α-hemolysin (6). Its biosynthesis is strongly induced by 1 mM free Ca^{2+} (7). Preliminary studies of the hemolytic activity of serotype 2 strain S1536 indicated that this strain must produce a different hemolysin, called hemolysin type II (HlyII) (7). The biosynthesis of this hemolysin is not induced by Ca^{2+}, but this ion is a cofactor for its hemolytic activity. Under optimal conditions, the hemolytic activity of serotype 2 strain S1536 (HlyII) in the culture supernatant was measured to be approximately 100 times lower than that found in supernatants of the HlyI-producing strain 4074 (7). Both HlyI and HlyII must be secreted efficiently, since a large fraction of their activity is found in the supernatants of bacterial cultures.

The aim of the present study was to analyze the regulation and the types of hemolysin found in the reference strains of all 12 serotypes and additional field isolates. We can classify the different serotypes of *A. pleuropneumoniae* into three groups: in the first group the very active and Ca^{2+}-regulated hemolysin HlyI is produced, in the second group the weaker hemolysin HlyII is produced, and in the third group both HlyI and HlyII are produced.

MATERIALS AND METHODS

Bacterial strains. The bacterial reference strains used in this study are listed in Table 1.

Chemicals, growth media, Ca^{2+} concentrations, and growth conditions. The following liquid medium was used unless otherwise mentioned: Supplemented Columbia broth (BBL Microbiology Systems, Cockeysville, Md.) supplemented with 1% IsoVitaleX (BBL) and 10 μg of β-NAD (Sigma Chemical Co., St. Louis, Mo.) per ml.

Complementation of the media with $CaCl_2$ (E. Merck AG, Darmstadt, Federal Republic of Germany) and determination of the free Ca^{2+} in the media were performed as described earlier (7). Depletion of Ca^{2+} was achieved by chelation with ethylene glycol-bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid (EGTA) (7). Proteinase K (protease type XI) was purchased from Sigma.

The *A. pleuropneumoniae* strains (except strains S1421 and 8329) were pregrown on supplemented Columbia agar plates at 37°C overnight. This culture was used to inoculate supplemented Columbia broth and then grown at 37°C to an A_{650} of 0.8 (midexponential growth), when the culture was used for hemolysin determinations. Pregrowth of the serotype 3 strain S1421 and serotype 12 strain 8329 was made on PPLO (20) agar plates, since pregrowth on supplemented Columbia agar plates resulted in a long lag phase and flocculation in the liquid growth phase.

Hemolysin titration. Hemolysin titrations were performed as described previously (6). Dilution series of the supernatant of cells grown in liquid medium to an A_{650} of 0.8 were

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TABLE 1. *A. pleuropneumoniae* reference strains

Strain	Serotype	Reference(s)
4074	1	14, 27
S1536	2	14
S1421	3	14
M62	4	14
K17	5a	14, 24
L20	5b	14, 24
femo	6	30
WF83	7	30
405	8	26
CVI13261	9	22
13039	10	23
56153	11[a]	11
8329	12	25

[a] Erroneously described as serotype 10 and tentatively classified as serotype 11.

made in TS buffer (10 mM Tris hydrochloride, 0.9% NaCl [pH 7.5]). The addition of $CaCl_2$ to this buffer is mentioned specifically when carried out. Samples (1 ml) of these dilutions were then mixed with 1 ml of 1% sheep erythrocytes in the same buffer, incubated for 2 h at 37°C, and then sedimented at 4°C overnight. The A_{540} of the supernatants was measured. Nonhemolytic controls contained equal volumes of buffer or growth medium and erythrocyte suspensions. One hemolytic unit (HU) was defined as the amount of material that lysed 50% of the sheep erythrocytes in 2 ml of a 0.5% suspension under the assay conditions described above.

Polyclonal antibodies and sera. Polyclonal antibodies directed against the purified HlyI (105-kDa protein) were produced in rabbits as described previously (8). Serotype 1-positive field sera from infected pigs were obtained from S. Larivière, Ste. Hyacinthe, Canada. Serotype 2-positive pig serum from an experimentally infected pig was obtained from R. Nielsen, Copenhagen, Denmark. Serum from a healthy pig that showed no reaction against *A. pleuropneumoniae* as tested by complement fixation test was used as the seronegative pig serum.

Neutralizations and immunoblot analysis. Purified HlyI diluted in TS buffer to an activity of 50 HU/ml or supernatants of the different cultures were mixed with polyclonal antibodies against HlyI or with sera from field-infected pigs. The sera were mixed with the supernatants at various dilutions and subsequently incubated for 30 min at 37°C for neutralization before the hemolytic activities were measured.

Proteins from total cultures grown to an A_{650} of 0.8 were separated for subsequent immunoblot analysis by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (12% acrylamide) as described by Laemmli (16). Samples of 20 μl at a protein concentration of 1.5 mg/ml were loaded per slot. Analysis by the immunoblot technique was as described by Towbin et al. (33).

RESULTS

Hemolytic activities of serotypes 1 through 12. The hemolytic activities of the reference strains of all 12 *A. pleuropneumoniae* serotypes (Table 1) were measured under four different conditions. (i) Cells were grown in low-Ca^{2+} (<10 μM, obtained by addition of 0.3 mM EGTA) medium followed by hemolysin titration without the addition of Ca^{2+} (to measure uninduced and Ca^{2+}-independent hemolysin). (ii) Cells were grown in low-Ca^{2+} medium, followed by hemolysin titration in buffer containing 10 mM $CaCl_2$ (to measure HlyII, which is dependent on Ca^{2+} for its activity). (iii) Cells were grown in high-Ca^{2+} (obtained by addition of 10 mM $CaCl_2$) medium followed by hemolysin titration in the absence of Ca^{2+} (to measure the Ca^{2+}-inducible HlyI). (iv) Cells were grown in high-Ca^{2+} medium, and hemolysin was titrated in buffer containing 10 mM Ca^{2+} (to measure the total hemolytic activity of both HlyI and HlyII). Figure 1 shows the hemolysin activity patterns of all 12 serotype reference strains. Cultures grown in low-Ca^{2+} medium and titrated in the absence of Ca^{2+} always had very low hemolytic activities. These activities could not be quantified accurately and must be below 1 HU/ml. The reference strains of serotypes 3 and 12 did not show any measurable hemolytic activities under all four conditions tested. The hemolytic activities of all serotypes were completely inactivated by incubation for 10 min at 60°C or by incubation for 10 min with 20 μg of proteinase K per ml for 10 min at 37°C.

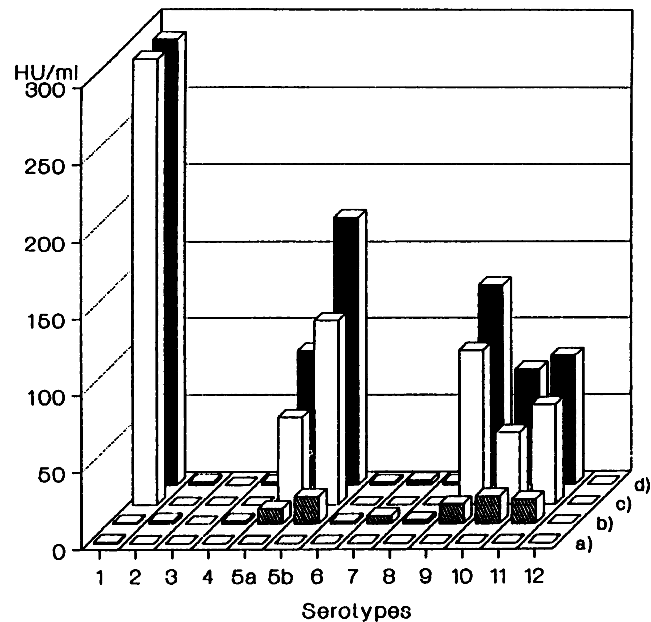

FIG. 1. Hemolysin pattern of the 12 serotypes of *A. pleuropneumoniae*. The hemolytic activities in supernatants of exponentially growing reference strains of the different serotypes were determined under the following conditions (rows): a, growth in low-Ca^{2+} medium and titration in the absence of Ca^{2+}; b, growth in low-Ca^{2+} medium and titration in the presence of Ca^{2+} (activity of HlyII is measured); c, growth in high-Ca^{2+} medium and titration in the absence of Ca^{2+} (activity of HlyI is measured); d, growth in high-Ca^{2+} medium and titration in the presence of Ca^{2+} (total hemolytic activity is measured).

Field isolates of two serotype 1 strains (5659 and 3933, from our laboratory), four serotype 6 strains (9175/88, 91380/88, 9185/88, and 9101 from R. Nielsen, Copenhagen), and two serotype 8 strains (8536/88 and 8932/88 from R. Nielsen) showed the same hemolysin profiles as their respective reference strains.

Neutralization of hemolysin. To verify the presence of two different hemolytic activities in the different serotypes, neutralization experiments were performed with rabbit antibodies against purified HlyI or sera of convalescent swine that had been infected with *A. pleuropneumoniae* serotype 1. The hemolytic activity of HlyI in supernatants of a culture of

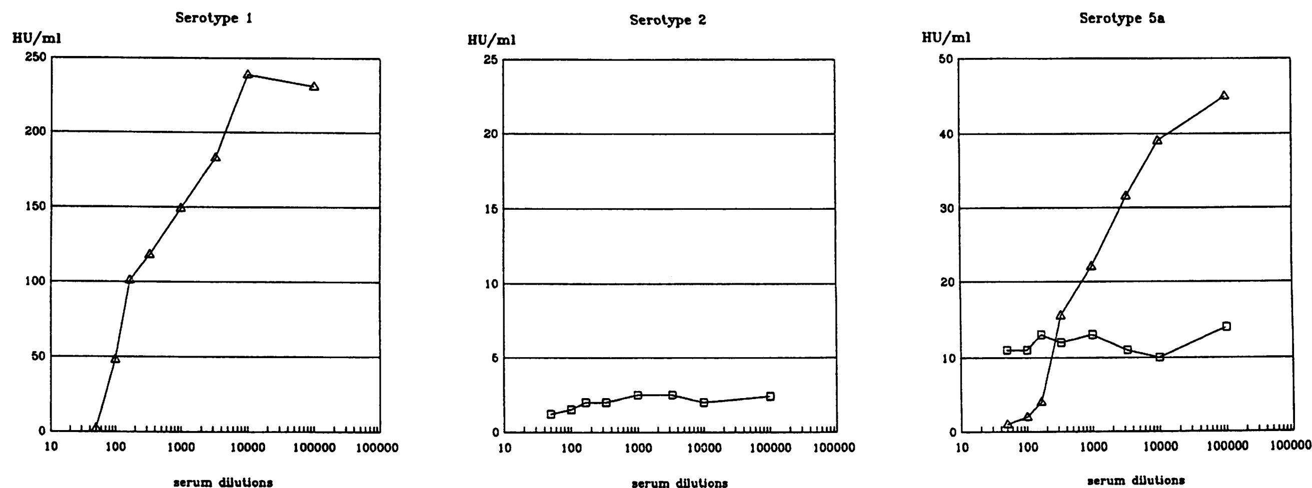

FIG. 2. Neutralization assays of the hemolytic activities in culture supernatants of serotypes 1, 2, and 5a. Rabbit anti-HlyI antibodies were used at the concentrations indicated. Symbols: △, hemolytic activities of cultures grown in high Ca^{2+} with titration in the absence of Ca^{2+} (HlyI); □, hemolytic activities of cultures grown in low Ca^{2+} with titration in the presence of Ca^{2+} (HlyII).

strain 4074 (300 HU/ml) or of purified HlyI (100 HU/ml) was completely neutralized by rabbit anti-HlyI antibodies at a dilution of 1/10. Complete neutralization was also obtained with convalescent-phase sera of pigs that were naturally infected with *A. pleuropneumoniae* serotype 1 at a concentration of 1/3. Preimmunization sera from rabbits or sera from pigs that were serologically negative for *A. pleuropneumoniae* or serum from pigs that were infected with serotype 2 neutralized neither the hemolytic activity of the supernatant of strain 4074 nor the purified HlyI. Pericardial and ascites fluid of necropsied pigs suffering from other conditions also were unable to neutralize HlyI.

Serum from a pig infected experimentally with serotype 2 neutralized the hemolytic activity at a dilution of 1/50 of the supernatant of cultures of the *A. pleuropneumoniae* strains that only express the HlyII activity (2 HU/ml), namely, S1536 (serotype 2), M62 (serotype 4), femo (serotype 6), WF83 (serotype 7), and 405 (serotype 8), but not of the other strains. Hemolytic activities of supernatants of these strains were also neutralized with sera from pigs infected with serotype 1 at the same dilution, but not with rabbit anti-HlyI (Fig. 2).

Neutralization assays with polyclonal antibodies directed against HlyI were performed on the culture supernatants of all 12 serotype reference strains and some field isolates that were grown in medium with low or high Ca^{2+}. The titrations of the hemolytic activities were done both in the presence of 1.5 mM Ca^{2+} and in its absence. Figure 2 shows the results obtained with reference strains of serotypes 1, 2 and 5a. The hemolytic activity in serotype 1 strain could be completely neutralized with anti-HlyI, whereas the hemolytic activity (HlyII) of serotype 2 was only partially neutralized. Results very similar to those found for serotype 2 were also found for serotypes 4, 6, 7, and 8 (data not shown). Neutralization experiments with serotype 5a indicated that the antibodies made against HlyI clearly neutralized the Ca^{2+}-inducible activity (HlyI) and neutralized the Ca^{2+}-dependent activity (HlyII) only slightly (Fig. 2). The same result was also obtained for the reference strains of serotypes 5b, 9, 10, and 11 (data not shown). These results are in agreement with the hemolysin patterns shown in Fig. 1, which indicate that these serotypes contain, in addition to HlyI, a second hemolytic activity, HlyII.

Immunoblotting. Samples of supernatants from cultures grown in Ca^{2+}-supplemented medium in midexponential growth (A_{650}, 0.8) were analyzed on immunoblots by using rabbit polyclonal antibodies directed against HlyI. The immunoblot shown in Fig. 3 shows reactions with a band at the same level as the 105-kDa HlyI protein for all the reference strains, indicating that these strains produce a protein that is antigenically related to HlyI. Some weak bands with lower molecular weights might be due to breakdown products. The reference strains for the serotypes 3 and 6 showed a very weak reaction on the immunoblot. This reaction, which could hardly be detected on some of the immunoblots, indicates that these two strains produce a protein that is only slightly related to HlyI or that is produced at a very low level.

DISCUSSION

To study the hemolysins secreted by the different strains and to evaluate their possible role in virulence, we analyzed the reference strains of all 12 serotypes of *A. pleuropneumoniae* as well as some field isolates, determining the type of hemolysin produced, its Ca^{2+} requirements for expression, and strength of the hemolytic activity. We have been able to distinguish two different hemolytic activities, HlyI and HlyII. These hemolytic activities differ in their regulation, their cofactor requirements, and their neutralization by specific antibodies. Both hemolytic activities are thermosensitive and are inactivated by protease. HlyI has been purified from serotype 1 strain and characterized as a 105-kDa protein (6). We were able to confirm its presence also in serotypes 5a, 5b, 9, 10, and 11 by neutralization experiments with polyclonal antibodies directed against the purified protein. HlyII has not been purified. The presence of HlyII in serotypes 2, 4, 5a, 5b, 6, 7, 8, 9, 10, and 11 is suggested by the Ca^{2+} dependence of the hemolysin activity. Its identity was confirmed by the lack of neutralization by specific antibodies to HlyI. (Surprisingly, it could be neutralized by sera from pigs infected naturally with serotype 1.) HlyII does not appear to be identical with the thermostable hemolysin of serotype 2 (15, 18). Indeed, we have not been able to detect thermostable hemolysins in any of the *A. pleuropneumoniae* reference strains with a sensitivity limit of 0.5

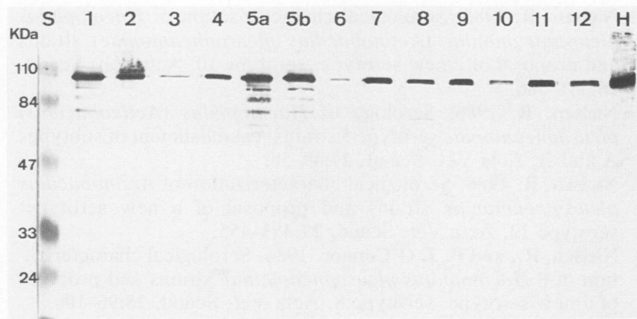

FIG. 3. Immunoblot of culture supernatants incubated with anti-HlyI. Supernatants of *A. pleuropneumoniae* reference strains for serotypes 1 through 12 and purified HlyI (lane H) was analyzed by immunoblot technique with rabbit antibodies against HlyI. The lanes are labeled according to the serotype number. Lane S contained prestained (no. 161-0305; Bio-Rad) molecular mass standards with the following apparent molecular masses: phosphorylase *b*, 110 kDa; bovine serum albumin, 84 kDa; ovalbumin, 47 kDa; carbonic anhydrase, 33 kDa; soybean trypsin inhibitor, 24 kDa.

HU/ml. Analyses of culture supernatants from all serotypes with specific rabbit antibodies to HlyI on immunoblots, showed in all strains a band of different intensity at a molecular weight similar to that of HlyI. Strains known to produce HlyI, with the exception of serotypes 2 and 7, showed the more intense reaction (Fig. 3). From these results it can be assumed that HlyI and HlyII are structurally and antigenically closely related but are different in their regulation and mode of activity. Recent DNA-DNA hybridization analysis done on chromosomal DNA from serotype 2 strain by using the cloned *hlyI* gene as a probe support this assumption (unpublished results).

The 12 serotypes of *A. pleuropneumoniae* can be classified in three different groups with respect to their production of hemolysins. Group 1 includes serotype 1 strains, which produce only the Ca^{2+}-inducible HlyI. Group 2 includes serotypes 2, 4, 6, 7, and 8, which produce only HlyII. A third group, including serotypes 5a, 5b, 9, 10, and 11, produces both types of hemolysins (HlyI and HlyII). Although serotypes 3 and 12 showed a faint hemolysis on blood agar plates, no hemolytic activity could be measured in the culture supernatants. These serotypes, however showed a faint band at 105 kDa on immunoblot analysis, indicating that they also produce a protein related to HlyI.

The hemolysin activity patterns shown in Fig. 1 demonstrate that, when present, HlyI constituted the major part of the activity of a strain, whereas the HlyII activity was much weaker. This effect is also observed when *A. pleuropneumoniae* strains are grown on blood agar plates. Recently, Kamp and van Leengoed (12), using the reference strains of serotypes 1 through 12, detected in culture supernatants of all serotypes except for serotype 6 a cytotoxic activity but a hemolytic activity only in serotypes 1, 5, 9, 10, and 11, which activity we assume to be due to HlyI in these strains. The conditions for measuring the hemolytic activity used by these authors, however, did not allow them to detect the relatively low activity of HlyII. It remains to be demonstrated whether the cytotoxic activity found by these authors in those serotypes (serotypes 2, 3, 4, 7, 8, and 12) for which they did not detect hemolytic activity could be due to the presence of HlyII.

It is interesting to note that all of the serotypes (serotypes 1, 5a, 5b, 9, 10, and 11) that produce HlyI are generally considered to be virulent types and are the serotypes that are frequently isolated from pigs in severe pleuropneumonia outbreaks (5, 17, 29; Nicolet, Ph.D. thesis). In a recent study of the virulence of the different serotypes with a mouse model, infection revealed that field isolates and reference strains belonging to serotypes 1, 5, 9, 10, and 11, which all produce HlyI, were highly virulent, whereas strains belonging to serotypes 2, 3, 4, 6, 7, 8, and 12, which do not produce HlyI, were significantly less virulent (J. P. S. Komal and K. R. Mittal, submitted for publication). These observations indicate that HlyI might be involved in the virulence of *A. pleuropneumoniae*.

In field situations, however, some serotypes, like 3, 7, and 8, that do not produce HlyI may be occasionally involved in severe outbreaks of pleuropneumonia (2, 4, 28). On the other hand, Rosendal et al. (31) deduced from experimental infections that serotypes 1, 2, and 7 were similarly virulent, whereas serotype 3 showed much lower virulence. Since it is known that differences in virulence may occur within the same serotype (1, 3, 10), it would be of great interest to determine the type of hemolysin secreted by field strains recognized as having low and high virulence. This should permit a better assessment of the role of hemolysins as determinants of virulence among other virulence attributes of *A. pleuropneumoniae*.

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