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Hemolytic Activity of the *Pasteurella haemolytica* Leukotoxin

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A *Pasteurella haemolytica* mutant incapable of producing leukotoxin was created by allelic replacement. Concentrated culture supernatants from wild-type *P. haemolytica*, but not from the mutant, contained the 102-kDa leukotoxin protein and lysed bovine lymphoma cells and sheep erythrocytes. Wild-type *P. haemolytica* demonstrated the typical beta-hemolytic phenotype on sheep and rabbit blood agar, whereas the mutant did not.

Pasteurella haemolytica, the causative agent of shipping fever pneumonia in cattle, was initially distinguished from other pathogenic members of the family *Pasteurellaceae* by its hemolytic phenotype (19). This hemolytic phenotype is associated with virulence for ruminant animals, but the gene conferring hemolysis has not been identified. Among the characterized virulence factors produced by *P. haemolytica* is a secreted, pore-forming cytolytic leukotoxin which has a target cell susceptibility limited to ruminant leukocytes and platelets (1, 2, 7, 21). This leukotoxin is a member of the RTX family of gram-negative bacterial toxins (23), and the genes encoding the *P. haemolytica* leukotoxin (LktA) and proteins necessary for its activation (LktC) and secretion (LktB and LktD) have been cloned and sequenced (10, 16, 22). As for most other members of the RTX toxin family, the four genes are adjacent to one another in a gene cluster (*lktCABD*) (Fig. 1a).

It has been suggested that the *P. haemolytica* leukotoxin is capable of lysing ovine erythrocytes (RBCs). Using *P. haemolytica* A1 culture supernatants or culture supernatants from recombinant *Escherichia coli* harboring *lktC*, *lktA*, and the *E. coli* genes *hlyB* and *hlyD*, others have demonstrated weak lysis of sheep RBCs (9). Highlander et al. (11) also reported small-zone hemolysis with similar *E. coli* recombinants after growth on sheep blood agar. The purpose of this study was to examine the role of *P. haemolytica* leukotoxin in RBC lysis. Utilizing a wild-type *P. haemolytica* strain and an isogenic strain with a mutated *lkt* genetic locus, we demonstrate that the *P. haemolytica* leukotoxin is lytic for both ruminant and rabbit RBCs.

We constructed a Lkt$^-$ mutant of *P. haemolytica* by allelic replacement, as shown in Fig. 1. The wild-type leukotoxin genetic locus spans an approximately 7-kbp region on the *P. haemolytica* chromosome (Fig. 1a). The mutated locus was constructed in vitro through a series of cloning steps. Briefly, an approximately 3,100-bp region of DNA, including the 3′ half of *lktC* and all but the final 54 bp of *lktA*, was replaced with an approximately 1,850-bp fragment carrying a *P. haemolytica* β-lactamase (*bla*) gene (Fig. 1b). A recombinant suicide plasmid, carrying this mutated locus, was introduced into *P. haemolytica* by electroporation as previously described (18). Transformants were selected on media containing ampicillin (10 μg/ml) and further analyzed by colony blot assay for hybridization to vector sequences (pACYC184), a DNA fragment within *lktA*, and the *bla* gene. One transformant (11-36) hybridized only to the *bla* gene probe (data not shown).

To determine the structure of the mutated *lkt* locus in this transformant, we examined chromosomal DNA by Southern blot hybridization with several DNA probes (Fig. 2). A 5.5-kbp *Hae*II fragment from the wild-type strain hybridized with a probe from within *lktA*. DNA from transformant 11-36 did not hybridize with this probe (Fig. 2a). A 4.3-kbp *Hae*II fragment from transformant 11-36 hybridized with the *bla* probe and with an *lktB* probe (*lktmut34*, Fig. 1) that consists of the 3′ end of *lktA* and a large segment of *lktB* (Fig. 2b and c). The *lktB* probe recognizes the 5.5-kbp *Hae*II fragment from the wild-type locus (Fig. 2b). These data are consistent with the allelic replacement of the wild-type *lktA* locus, as described above, through a double crossover homologous recombination event.

To verify that this strain was no longer capable of producing LktA, we examined concentrated culture supernatants (CCS) by Western immunoblot assay. Wild-type and LktA$^-$ *P. haemolytica* strains were grown under conditions conducive to leukotoxin production. Briefly, flasks containing 30 ml of RPMI medium were inoculated with the appropriate strain and grown from an A_{600} of 0.25 to 0.90, at 37°C on a rotary shaker (70 oscillations per min). Culture supernatants were collected by centrifugation, and each supernatant was concentrated to the same level by ammonium sulfate precipitation (0 to 60%; 361 g/liter). CCS from each strain were resuspended in 1.7 ml of 50 mM sodium phosphate (pH 7.0)–100 mM NaCl buffer, dialyzed against the same buffer, and stored at −135°C (5). The presence of the 102-kDa leukotoxin band in Western immunoblots of CCS from the wild-type and mutant strains was examined with a murine immunoglobulin G, subclass 2a, anti-leukotoxin monoclonal antibody (C6). The monoclonal antibody recognized the 102-kDa leukotoxin band and associated breakdown bands in CCS from the wild-type strain, but no leukotoxin band was detected in CCS from the mutant strain (Fig. 3).

We next compared the growth rates of the wild-type and LktA$^-$ strains, the production of lipopolysaccharide (LPS) and capsule by both strains, and the total protein concentration, leukotoxic activity, and hemolytic activity present in the CCS from both strains. Growth time from A_{600} of 0.25 to 0.9 was 2.5 h for both the wild-type and LktA$^-$ strains, with no observed difference in the growth rate ($n = 4, P > 0.05$; data not shown). Similarly, we observed no detectable differences in the O-antigen banding patterns of LPS or in the lower-molecular-

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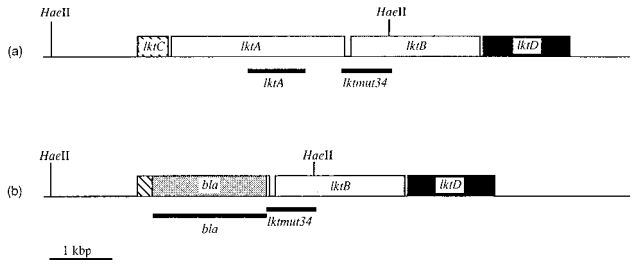

FIG. 1. Wild-type (a) and mutant (b) *lkt* genetic loci. Thin black bars represent nucleotide fragments used as probes in Southern blot analyses (Fig. 2).

mass LPS bands from the wild-type and LktA$^-$ strains (data not shown). Wild-type and LktA$^-$ strains were stained for capsule as previously described (8), and we detected no obvious differences between the strains. Both the wild-type and LktA$^-$ strains were encapsulated (data not shown).

Statistically significant differences ($n = 4$, $P < 0.05$) were observed for protein concentration and leukotoxic and hemolytic activities in wild-type versus LktA$^-$ CCS. The protein concentrations of the CCS for the wild-type and LktA$^-$ strains were 0.630 ± 0.028 and 0.481 ± 0.088 mg/ml, respectively (BCA microprotein assay, Pierce Chemical Co., Rockford, Ill.).

Leukotoxic activity was assessed with a standard assay measuring leakage of lactate dehydrogenase from cultured bovine lymphoma cells (BL3 cells), as described elsewhere (6). CCS from the wild-type strain exhibited leukotoxic activity at dilutions of 1:5 to 1:100, whereas no leukotoxic activity was detected for CCS from the LktA$^-$ strain (Fig. 4). Therefore, the wild-type *P. haemolytica* strain used here produces a 102-kDa protein identified as the leukotoxin by monoclonal antibody and exhibits leukotoxic activity compatible with the leukotoxin,

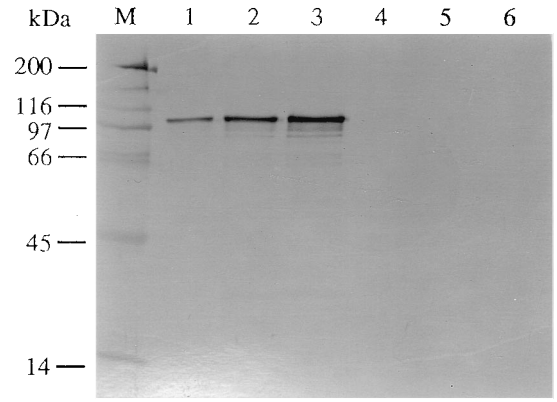

FIG. 3. Western immunoblot of CCS from wild-type and LktA$^-$ *P. haemolytica* probed with the anti-leukotoxin monoclonal antibody C6. Lanes: 1, 0.3 μg; 2, 1.6 μg; 3, 3.3 μg; 4, 0.2 μg; 5, 1.2 μg; 6, 2.4 μg. Lanes 1 to 3 contain CCS from wild-type *P. haemolytica*, and lanes 4 to 6 contain CCS from LktA$^-$ *P. haemolytica*. The 102-kDa LktA band is present in CCS from the wild-type strain but not from the mutant strain.

whereas the LktA$^-$ strain neither produces the leukotoxin nor demonstrates leukotoxic activity.

Hemolytic activity of the CCS was assessed by exposing 2×10^6 washed bovine RBCs to serially diluted CCS in 200 μl of RPMI for 2 h at 37°C, in 96-well plates. The amount of hemolysis effected by 100 μl of assay supernatants was then determined by measuring the optical density at 405 nm (OD_{405}) of the released hemoglobin. Maximal hemolysis was determined as the amount of hemoglobin released by treatment of RBCs with 0.1% Triton X-100, and background hemolysis was determined by substitution of buffer or concentrated uninoculated media for CCS. The assay supernatant OD_{405} for the 1:5 dilution of the wild-type CCS was statistically different ($n = 4$, $P <$

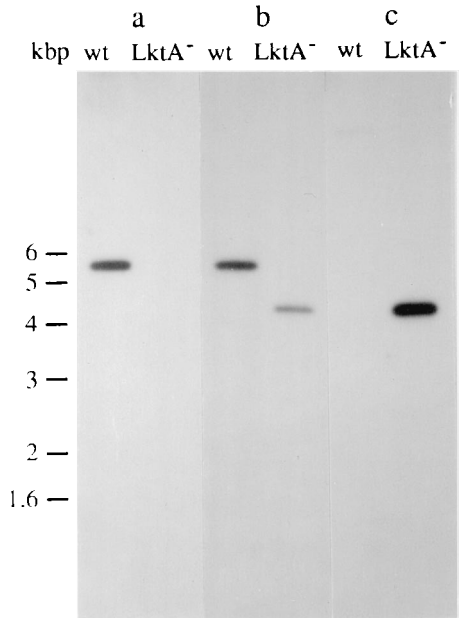

FIG. 2. Southern blot analyses of *Hae*II-digested DNA from wild-type (wt) and LktA$^-$ *P. haemolytica* probed with an *lktA* fragment (a), an *lktB* fragment (*lktmut34*) (b), and a *bla* fragment (c). The positions of the genetic probes are shown in Fig. 1. Positions of size standards are shown on the left.

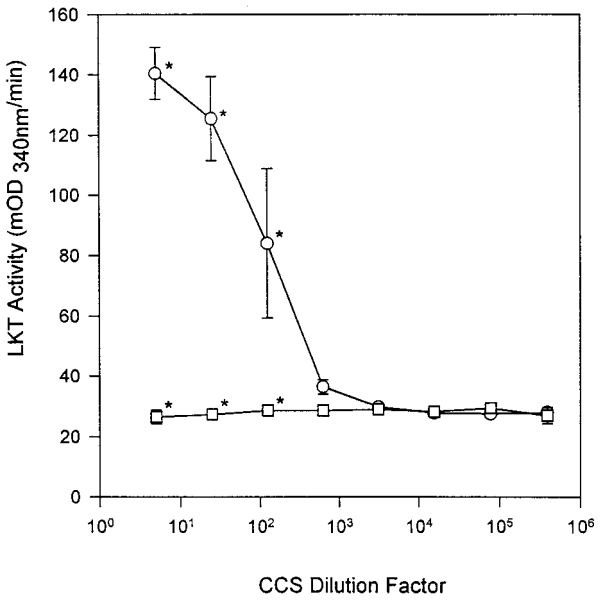

FIG. 4. Leukotoxic activity of CCS from wild-type (○) and LktA$^-$ (□) *P. haemolytica* against BL3 cells. Activity is expressed as 10^{-3} OD units (mOD) per minute per 100 μl. Triton X-100 released 224×10^{-3} OD units/min/100 μl from BL3 cells. Lactate dehydrogenase leakage values which are significantly different (t test with $P < 0.05$, $n = 4$) for the same dilution of CCS from wild-type and LktA$^-$ strains are indicated (*). The error bars indicate the standard deviation.

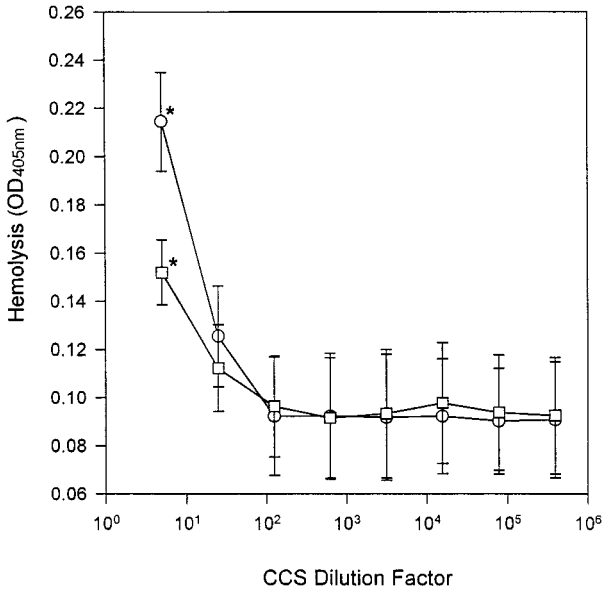

FIG. 5. Hemolytic activity of CCS from wild-type (○) and LktA$^-$ (□) *P. haemolytica* against washed bovine RBCs. Hemoglobin leakage is expressed as OD_{405} units per 100 μl of assay supernatant. Treatment of RBCs with Triton X-100 released 0.565 OD_{405} unit. Hemoglobin leakage values which are significantly different (*t* test with $P < 0.05$, $n = 4$) for the same dilution of CCS from wild-type and LktA$^-$ strains are indicated (*). The error bars indicate the standard deviation.

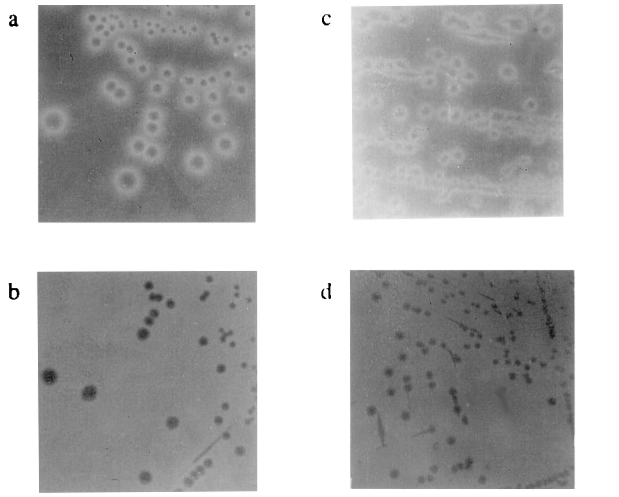

FIG. 6. Hemolysis of sheep (a and b) or rabbit (c and d) RBCs after growth of the wild-type (a and c) and LktA$^-$ (b and d) strains on blood agar plates. The growth medium was prepared with blood agar base (Difco) and 5% sheep or rabbit blood.

0.05) from that for the LktA$^-$ CCS, but no significant differences were observed at any of the higher dilutions (Fig. 5). The percent specific hemolysis was calculated as (CCS OD_{405} − blank OD_{405})/(Triton X-100 OD_{405} − blank OD_{405}) × 100, and the values were 19.2 and 4.6% for the 1:5 dilution of the wild-type and LktA$^-$ CCS, respectively. The percent specific hemolysis caused by concentrated uninoculated media was similar to that caused by CCS from the LktA$^-$ strain. Therefore, the low level of hemolysis observed for the mutant is most likely due to concentrated medium components. The hemolytic activity of wild-type *P. haemolytica* CCS is low compared with that of other hemolytic RTX cytolysins. For hemolysis detection, it was necessary to reduce the concentration of RBCs from 1 to 0.04% (17).

We also examined beta-hemolysis after growth of the wild-type and LktA$^-$ strains on agar containing washed sheep or rabbit RBCs (Fig. 6). In both cases, growth of the wild-type strain was accompanied by a definite zone of hemolysis surrounding colonies (Fig. 6a and c), whereas no zone of clear hemolysis was associated with growth of the LktA$^-$ strain on these plates (Fig. 6b and d).

Our results demonstrate that the *P. haemolytica* leukotoxin possesses a broader target cell specificity than previously thought. As mentioned earlier, previous studies on culture supernatants from *P. haemolytica* and from recombinant *E. coli* carrying parts of the leukotoxin genetic operon suggested that ovine RBCs were susceptible to leukotoxin. Here we have shown that the leukotoxin is capable of lysing RBCs from ruminants and a nonruminant species. These results also suggest that LktA is the gene product responsible for the beta-hemolytic phenotype on rabbit blood agar, for which *P. haemolytica* was given its taxonomic name.

Others have recently reported the isolation of *P. haemolytica* leukotoxin mutants after nitrosoguanidine mutagenesis (4). Those authors reported no alterations in the hemolytic activity of two such mutants on 5% sheep blood agar plates. Hemolysis was observed upon removal of the colonies from the agar plate; however, those data were not shown. The basal medium used for the preparation of those agar plates is not readily apparent. We have found that wild-type *P. haemolytica* exhibits pronounced beta-hemolysis when blood agar base (Difco Laboratories, Detroit, Mich.) is used as a medium for the preparation of ovine (Fig. 6) or bovine blood agar plates, whereas the LktA$^-$ strain is clearly nonhemolytic (Fig. 6) on sheep blood agar plates prepared in this manner. We also examined beta-hemolysis using brain heart infusion as a basal medium for blood agar plates and observed the same results. It is possible that the parental strain of *P. haemolytica* used by Chidambaram et al. (4) possesses some other form of hemolytic activity, thus accounting for the differences in our observations.

A conceivable role for this hemolytic activity in the pathogenesis of pneumonic pasteurellosis would be in the acquisition of iron from the bovine host. Bovine hemoglobin has previously been shown to enhance the virulence of *P. haemolytica* in a mouse model of infection (3) through an as-yet-undescribed mechanism. Citing other work (12), those authors suggested that iron may inhibit leukocytic killing of *P. haemolytica* after the organism has bound the free iron through an iron-binding protein. Others have demonstrated that *P. haemolytica* is able to use bovine hemoglobin as a source of iron for growth (20). In that study, hemoglobin did not block binding of transferrin to iron-deficient *P. haemolytica*, and siderophore production by *P. haemolytica* was not detected. Therefore, *P. haemolytica* may produce a hemin-binding protein that allows for acquisition of heme-iron. Such receptors have recently been identified in *Neisseria meningitidis* (15), *Neisseria gonorrhoeae* (13), and *Haemophilus influenzae* type b (14) and shown to be distinct from the transferrin-binding protein produced by those organisms. The identification of such a receptor in *P. haemolytica* would allow for an evaluation of the role of leukotoxin in iron acquisition.

The mutant described here harbors specific changes to a single genetic locus. This strain is a useful control for studies which examine the effects of leukotoxin on bovine host cells. The mutant will also be useful in studies on the pathogenesis of *P. haemolytica*-induced pneumonic pasteurellosis, by allowing

us to more effectively examine the interactions of bovine neutrophils and macrophages with the bacterial cell.

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