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Isolation of *Pasteurella haemolytica* Leukotoxin Mutants

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Two mutants of *Pasteurella haemolytica* A1 that do not produce leukotoxin were isolated. Following mutagenesis, colonies were screened with antiserum by a filter assay for absence of the secreted leukotoxin. The two mutants both appeared to produce normal amounts of other antigens, as judged by reactivity with polyclonal serum from an animal with pasteurellosis, and were not altered in beta-hemolytic activity as seen on blood agar plates. There was no evidence of either cell-associated or secreted leukotoxin protein when Western blots (immunoblots) were carried out with the polyclonal serum or with a monoclonal antibody directed against the leukotoxin. Southern blots revealed that both mutants show the wild-type restriction pattern at the leukotoxin locus, although the strain with the *lktA2* mutation showed differences in other regions of the chromosome on analysis by pulsed-field gel electrophoresis. The strain with the *lktA2* mutation grew more slowly than did the wild-type strain, while the strain with the *lktA1* mutation was indistinguishable from the wild-type strain in its growth properties. The strain with the *lktA1* mutation should be valuable in determining the role of the leukotoxin in virulence as well as in identifying other virulence factors of *P. haemolytica*.

Bovine pneumonic pasteurellosis (shipping fever), usually due to infection by *Pasteurella haemolytica* biotype A serotype 1 (4, 33), is a major disease of feedlot cattle, resulting in significant economic losses (24). Although *P. haemolytica* is widely carried by cattle, it appears to cause serious disease only when the animal's normal immune mechanisms are impaired by stress, viral infections, or other factors. Studies of *P. haemolytica* have identified a number of potential factors that may contribute to virulence and/or which could be used to control infections or pathogenesis. These factors include a secreted leukotoxin (21, 29), capsular polysaccharide (2, 13), outer membrane proteins (31), fimbriae (25), secreted sialylglycoprotease (1), secreted neuraminidase (12, 32), type-specific antigen (15), and lipopolysaccharide (9). One goal of research in this area is to determine which of these putative virulence factors are the best targets to exploit in development of new effective therapeutics or vaccines.

One approach used to evaluate the significance of the various components of *P. haemolytica* in infection has been to determine if immunization with a given protein results in protection from challenge infection. Vaccines using *P. haemolytica* elements without significant leukotoxin antigen have been of variable efficacy (3, 5–8, 11, 26, 30). However, addition of leukotoxin to a vaccine has improved its effectiveness (10). We have also demonstrated that, under certain conditions, immunization with leukotoxin alone can give protection against *P. haemolytica* infection (36). Observations such as these have led to the notion that leukotoxin is one of the key virulence factors in *P. haemolytica* infection. The presumed role of the leukotoxin is destruction of leukocytes at the site of infection, which thus reduces the animal's capacity to launch an effective immune response. The lysis of immune cells by the toxin also results in release of destructive enzymes into the lung tissue, contributing to the severe necrosis observed in *P. haemolytica* infections.

An approach complementary to immunization studies that has been successful in studying virulence of numerous pathogens is the construction of mutant strains deficient in one or more purported virulence factors and comparison of the mutants' virulence with that of the wild-type parent. Such an approach avoids some of the complications of vaccine studies. In addition, such mutant strains can be used to study the role of virulence components that might otherwise be masked by the presence of other factors. With this in mind, we report here the isolation and initial characterization of two mutants of *P. haemolytica* that do not produce leukotoxin. In the accompanying paper (27), we report their antigenic and virulence properties.

MATERIALS AND METHODS

Bacteria and plasmids. *P. haemolytica* 59B049, the parent of the mutants, is a serotype A1 strain originally isolated from a pneumonic bovine lung. *P. haemolytica* PHL101, used in an earlier study (16) to clone the *lkt* locus, was obtained from G. H. Frank, National Animal Disease Center, Ames, Iowa. Strain PHL101 was also the source of DNA for construction of a cosmid clone (19) containing the entire *lktCABD* locus, as well as 8 kb of *P. haemolytica* chromosomal DNA flanking this locus. This clone was used as the probe in Southern blots of mutants.

Reagents. Serum obtained from cattle recovering from shipping fever was provided by Cactus Feeders, Inc. (Amarillo, Tex.). This serum, designated anti-PH serum, previously has been shown (16) to contain antibodies against a variety of *P. haemolytica* antigens, including the leukotoxin. Bovine serum directed specifically against the leukotoxin was prepared by immunizing animals with leukotoxin antigen purified from an *Escherichia coli* strain overproducing this polypeptide (16, 17). Unlike the serum from convalescent cattle, this serum specifically reacts only with the leukotoxin polypeptide and not with other *P. haemolytica* antigens (36). This serum was used to screen colonies for mutants. Monoclonal antibody (MAb) MM601 is a leukotoxin-neutralizing antibody (14) obtained from S. Srikumaran, University of Nebraska. Markers for protein gels were prestained protein molecular weight standards from GIBCO-BRL (Gaithersburg, Md.), or biotinylated molecular mass standards from Bio-Rad (Richmond, Calif.).

Growth of *P. haemolytica*. Brain heart infusion (BHI) broth (Difco Laboratories, Detroit, Mich.) was used for routine liquid growth of *P. haemolytica*, and 5% sheep blood agar plates were used for routine plating. Overnight cultures of *P. haemolytica* were prepared by inoculating BHI broth with cells from a fresh blood agar plate and incubating at 37°C. Exponentially growing cultures were

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prepared from overnight cultures by making a 1:50 dilution and incubating until the cell density reached 150 Klett units, approximately 5×10^8 cells per ml. For some studies, an exponentially growing culture (5×10^8 cells per ml) instead of a culture grown overnight to saturation was diluted 1:10 to start a new culture with no lag period.

Phage detection. To test for the presence of the lysogenic phage carried by *P. haemolytica* strains (28), cell lysis induced by mitomycin C (Sigma Chemical Co., St. Louis, Mo.) was measured. We have previously verified by physical isolation and electron microscopy that mitomycin C induces the *P. haemolytica* lysogenic phage (37). Cells were grown in BHI broth to early log phase (about 1.5×10^8 cells per ml) and then induced by adding mitomycin C to 1 μg/ml. The culture was allowed to incubate with aeration for 3 h, at which time lysis was observed.

Mutagenesis with NTG. A fresh stock solution of nitrosoguanidine (NTG) was prepared by dissolving NTG (Sigma) in 100% ethanol and diluting with sterile water to a concentration of 1 mg/ml. The *P. haemolytica* culture to be mutagenized was grown in BHI broth to 5×10^8 cells per ml, centrifuged, washed twice, and finally resuspended in citrate-phosphate buffer (0.93% citric acid, 0.11 M sodium phosphate dibasic [pH 5.2]). The resuspended cells were dispensed into a microcentrifuge tube, and 10 μg of NTG per ml was added. The mixture was incubated at 37°C for 30 min, at which time the reaction was stopped by washing with 0.1 M phosphate buffer (pH 7.0). Survival was monitored by plating cells on either BHI or blood agar plates. An overnight incubation was used to allow phenotypic expression of mutations. A 0.1-ml aliquot of the mutagenized cells was diluted into 10 ml of BHI broth, and the mixture was shaken vigorously at 37°C for 16 h. To measure mutagenesis, the frequency of nalidixic acid-resistant mutants in these overnight cultures was measured by plating cells on BHI agar plates containing 20 μg of nalidixic acid per ml. To identify defective leukotoxin producers, cells were spread on 5% blood agar plates and a sterile nitrocellulose filter (Schleicher & Schuell, Inc., Keene, N.H.) was carefully placed on top of the cells. The plates were incubated at 37°C for approximately 48 h. The colonies grew underneath the filters, and most cells stuck to the filters when the filters were peeled off. A zone of hemolysis was clearly visible where the colonies had grown. The filters were processed with antibody as described below to detect leukotoxin production. After removal of the filters and screening for any alterations in hemolysis, the blood agar plates were reincubated to allow the colonies to reform from the cells remaining on the plates.

Screening for leukotoxin mutants. The nitrocellulose filters that had been laid over the mutagenized colonies were washed carefully in TBS (10 mM Tris [pH 7.6], 0.9% NaCl) and then incubated in TBS containing 2% nonfat milk (Carnation or Bio-Rad) for 60 min. This procedure washed the cells off the filter, but any protein that had been secreted remained bound. Bovine antiserum directed against leukotoxin (but not against other *P. haemolytica* antigens) was then added at a 1:500 dilution, and the filters were incubated at 4°C overnight with gentle shaking. Typically, 0.2 ml of serum was added to a 100-ml mixture with 10 filters. After incubation, the filters were washed five times with TBS and subsequently incubated with biotin-labeled goat anti-bovine immunoglobulin G (1:500 dilution in 100 ml of TBS; Kirkegaard & Perry Laboratories, Gaithersburg, Md.), washed again, and then incubated in 100 ml of TBS with horseradish peroxidase-conjugated streptavidin (1:1,000 dilution; Kirkegaard & Perry Laboratories). Bound horseradish peroxidase was detected by incubating the filters with hydrogen peroxide in the presence of 4-chloro-1-naphthol (Sigma Chemical Co.). When the stained filters were compared with the colonies on the blood agar plates, it could be seen that this treatment led to a purple stain on the filter at the position of each colony. The absence or reduction of color development indicated a mutant with a defect affecting the production or secretion of leukotoxin antigen by cells in the colony. Putative mutants were restreaked on blood agar, covered with filters, and retested to confirm the leukotoxin phenotype.

Antimicrobial susceptibilities. Susceptibilities of the parent strain and mutant 59B0071 to a broad panel of antimicrobial agents were assessed by microtiter dilution methods (20). Antimicrobial agents were from commercial suppliers or were obtained from Central Research Division, Pfizer Inc. The antimicrobial agents were solubilized in appropriate solvents, and serial twofold dilutions were made in BHI broth. The inoculum was prepared by using the BBL Prompt Inoculation System (3M) according to manufacturer's instructions. Microtiter wells were inoculated to a cell density of approximately 10^5 CFU/ml. Following incubation at 37°C for 16 to 18 h, A_{600} was read by using a Biotek EL320 microplate reader. The MIC was defined as the lowest concentration of drug which inhibited growth of *P. haemolytica* to an A_{600} value of ≤0.025.

Western blots. For Western blots (immunoblots) with anti-PH serum, late-exponential-phase cultures (about 8×10^8 cells per ml) were centrifuged to separate cells and supernatants. Each fraction was mixed with 3× tracking dye, boiled, and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as described elsewhere (16). After electrophoresis, the proteins were transferred to Immobilon filters (Millipore Corp., Bedford, Mass.) and probed (16) with polyclonal anti-PH serum at dilutions of 1:250 to 1:500. The antigens were detected by the Western blot method of Towbin et al. (34).

For Western blot analysis with MAb MM601, supernatants from cultures were filtered through 0.22-μm-pore-size Durapore filters (Millipore) and then concentrated approximately 10-fold with a Centricon ultrafiltration device with a molecular weight cutoff of 30,000 (Amicon, Beverly, Mass.). Concentrated supernatants were mixed with 2× Laemmli sample buffer (22) and boiled for 5 min. Whole-cell lysates were prepared by washing the harvested cells three times with fresh BHI broth, resuspending them in 10 volumes of 50 mM Tris (pH 7.5)–10 volumes of 2× Laemmli sample buffer, and boiling for 5 min. Proteins in culture supernatants and cell lysates were separated by SDS-PAGE as described above, electroblotted onto nitrocellulose filters, and probed with MM601 (1:40,000 dilution). Antigens were detected by using peroxidase-conjugated goat anti-mouse immunoglobulin G (1:5,000 dilution; Kirkegaard & Perry Laboratories) and an ECL enhanced chemiluminescence detection system (Amersham, Arlington Heights, Ill.). Biotinylated molecular mass standards from Bio-Rad were used and were detected with peroxidase-conjugated streptavidin (1:5,000 dilution; Kirkegaard & Perry Laboratories).

DNA manipulations. Bacterial DNA was prepared for digestion with restriction endonucleases by a procedure using cells embedded in agarose plugs. *P. haemolytica* was grown in BHI broth to about 7×10^8 cells per ml, and 10 ml of the cells was harvested by centrifugation and resuspended in 2.0 ml of PIV (10 mM Tris, 1 M NaCl). An equal volume of 1.6% Incert agarose (FMC Corp., Rockland, Maine) was added to the cell suspension, and then the mixture was warmed to 37°C, poured into a plug mold (Bio-Rad), and allowed to harden. The plugs were incubated overnight at 37°C in EC lysis buffer (6 mM Tris, 1 M NaCl, 0.1 M EDTA, 0.5% Brij, 0.2% deoxycholate, 0.5% Sarkosyl, 20 μg of RNase per ml, 1 mg of lysozyme per ml). The EC lysis buffer was carefully decanted, and the plugs were incubated in ESP (0.5 M EDTA [pH 9.0], 1% Sarkosyl, 50 μg of proteinase K) at 50°C overnight with gentle shaking. The plugs were then washed three times with TE (10 mM Tris, 0.1 mM EDTA) and stored in the cold. For resolution of large restriction fragments, chromosomal DNA embedded in agarose was digested with *Not*I (Promega, Madison, Wis.) or *Asc*I (New England Biolabs, Beverly, Mass.) or sequentially digested with the two enzymes at 37°C. Most digests were allowed to incubate overnight, and then plugs were rinsed with TE. The digested plugs were loaded on a 1% Fastlane agarose (FMC) gel in 0.25% TBE (23). The gel was run in a CHEF DRII gel apparatus (Bio-Rad). Different pulse times were used to optimize the separation of fragments. The gels were stained with ethidium bromide and photographed under UV illumination. Transfer of fragments from contour-clamped homogeneous electric field (CHEF) gels to membranes for Southern blot analysis was done by the procedure described in the Bio-Rad CHEF gel apparatus manual. For resolution of smaller fragments, DNA in agarose blocks was similarly digested with *Bgl*II, *Xba*I, *Pst*I, or *Cla*I but run in standard agarose gels. A multiprimer labeling system (Amersham) was used to label the *lktCABD* cosmid DNA as a probe for Southern blots.

RESULTS

Strategy for isolating mutants with mutations affecting leukotoxin production. To identify mutants with a defect in leukotoxin production, an immunological screening procedure was developed. *P. haemolytica* colonies were grown on blood agar plates covered with a nitrocellulose filter. Under these conditions, the cells grow well and secrete the leukotoxin protein. The secreted leukotoxin (and presumably any other secreted proteins) sticks to the nitrocellulose filter and can be detected by a standard colorimetric immunoassay. The antiserum used for immunodetection in this study was specific for the leukotoxin and did not react with other *P. haemolytica* antigens. Thus, each colony on the blood agar plate generates a signal on the filter due to the secreted leukotoxin. Mutants that fail to synthesize leukotoxin can be identified as colonies on the blood agar plate that produce little or no signal on the filter. Two such mutants were found. Mutants that synthesize leukotoxin but fail to secrete it from the cell could also show this phenotype, but no mutants in this class were found.

Isolation of mutants. Since no mutagenesis procedure had been published previously for *P. haemolytica*, it was first necessary to establish a mutagenesis protocol. One complication was the fact that this bacterium carries a prophage that is induced by many DNA-damaging treatments (28). Thus, any mutagen that was used should avoid prophage induction. NTG, a potent mutagen that is not as efficient at prophage induction as other mutagens, was chosen for these reasons.

To establish mutagenesis conditions, we initially isolated mutants with mutations conferring nalidixic acid resistance. This phenotype was found to occur spontaneously at low frequency ($\sim 10^{-8}$) with *P. haemolytica*, and there was little background growth of sensitive cells on plates containing 20 μg of nalidixic acid per ml. Thus, this type of mutant provided a convenient method to establish conditions for NTG mutagen-

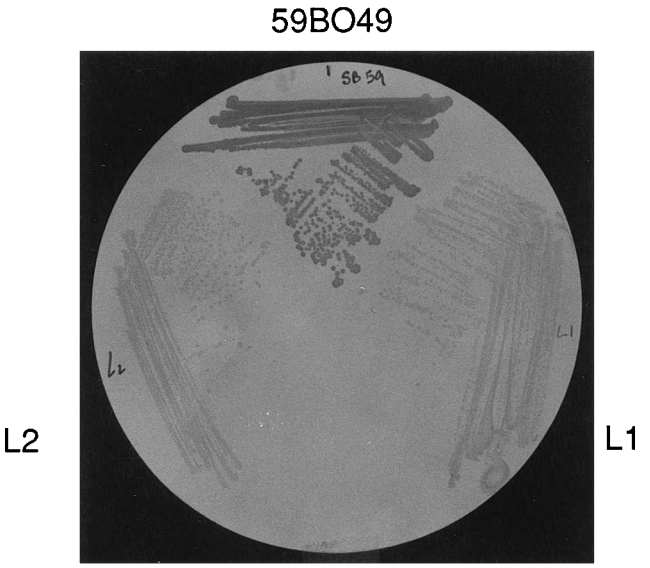

FIG. 1. Immunostaining of wild-type and mutant *P. haemolytica* strains. The wild-type parent (59B049 *lktA*$^+$) and the two mutants 59B0071 and 59B0072 (alleles *lktA1* and *lktA2*) were streaked on a blood agar plate, covered with a nitrocellulose filter, and incubated overnight. The next day, the filter was removed and incubated with antiserum as described in Materials and Methods. Shown is the filter after immunostaining. L1, strain 59B0071; L2, strain 59B0072.

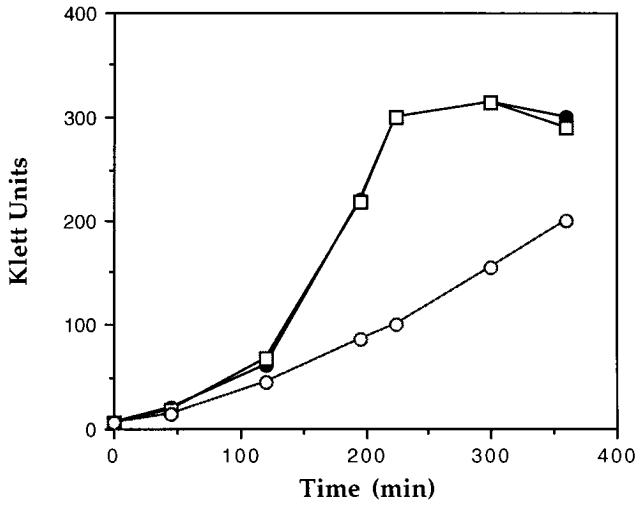

FIG. 2. Growth of wild-type and mutant *P. haemolytica* strains. The wild-type parent (59B049, filled circles) and the two mutants (59B0071, open squares, and 59B0072, open circles) were grown in BHI broth as described in Materials and Methods.

esis (see Materials and Methods). Under these conditions, the cell survival rate after mutagen treatment was relatively high (typically 5 to 10%) and mutagenesis to nalidixic acid resistance was stimulated at least 50-fold.

Following this mutagenesis procedure, approximately 30,000 colonies were screened for defects in leukotoxin production. Two mutants were obtained and are described below. In addition, several other classes of phenotypes were observed. The most striking class (10 to 20 isolates) were colonies that were white or silver on blood agar plates. These colonies showed poor staining on the filters treated with antileukotoxin antibodies. However, when these mutants were analyzed by Western blots they were found to produce and secrete normal levels of leukotoxin. These mutants may bear mutations affecting synthesis of capsule or in some other way interfering with the immunoassay. In addition to screening for leukotoxin-deficient mutants by the immunoassay, we also examined the blood agar plates for mutants with altered hemolytic activity. No mutants with grossly increased or decreased activity were observed.

Characterization of mutants. The two mutant strains obtained, 59B0071 and 59B0072 carrying the *lktA1* and *lktA2* alleles, respectively, both showed a reduction, rather than a complete elimination, in color development in the filter assay (Fig. 1). As shown below, neither of these mutants produces leukotoxin. Thus, this background immunoreactivity is independent of leukotoxin. Using this assay, we have observed an activity in *P. haemolytica* that gives a colorimetric reaction in Western blots even when antibody against *P. haemolytica* antigens is omitted. This may be a streptavidin-binding activity or a peroxidase. In either case, this activity most likely accounts for the leukotoxin-independent background in the filter assay. Despite this, the mutant leukotoxin-negative immunostaining phenotype was easily distinguishable from the wild-type phenotype (Fig. 1). We assessed the stability of the phenotypes by plating out several thousand mutant cells and screening the resulting colonies with antibody. No wild-type revertants were seen. Thus, we conclude that the mutants are stable, with a reversion frequency of less than 10^{-3}.

The growth of the mutant strain 59B0071 (*lktA1* allele) was identical to that of the parent strain, whereas strain 59B0072 (*lktA2* allele) grew at a significantly reduced rate (Fig. 2). Both mutants and the parent were found to harbor the prophage (28), as judged by the kinetics of lysis after treatment with mitomycin C (data not shown). Metabolic profiles of strain 59B0071 and the parent showed no differences by the API 20E system; slow growth of strain 59B0072 precluded a direct comparison with the other strains. Antimicrobial susceptibilities for strains 59B0071 and 59B049 (the wild-type parent) were identical or within 1 twofold dilution for the following drugs: amikacin, ampicillin, azithromycin, bacitracin, bicyclomycin, cefuroxime, cephaloridine, cerulenin, chloramphenicol, colistin, efrotomycin, enrofloxacin, erythromycin, fusidic acid, gentamicin, globomycin, kanamycin, lincomycin, methicillin, moenomycin, monensin, nalidixic acid, norfloxacin, novobiocin, oxytetracycline, paromomycin, K penicillin G, rifampin, spectinomycin, spiramycin, streptomycin, tetracycline, thiolactomycin, tiamulin, tobramycin, trimethoprim, tylosin, and vancomycin. Polymyxin B showed a fourfold MIC differential although both strains were highly susceptible. These results suggest that there are no major alterations in cell wall or membrane structure or physiological processes that affect susceptibility to structurally and mechanistically diverse antimicrobial agents.

Western blots with serum (obtained from an animal recovering from pasteurellosis) that contained antibodies against a variety of *P. haemolytica* antigens (anti-PH serum) and a leukotoxin-neutralizing MAb were used to compare the parent strain with the two mutants. The leukotoxin protein appears as a band of approximately 102 to 104 kDa in the parent strain 59A049; none of this protein is evident in the supernatants of either of the mutants when the anti-PH serum (Fig. 3A, lanes 1 and 2) or MAb MM601 (panel B, lanes 1 and 3) is used. The anti-PH serum also reveals a large reduction in cell-associated antigenic material at the molecular weight of the leukotoxin in the mutants, although a faint band is present that appeared to migrate slightly more slowly than the leukotoxin. That this may be a nonleukotoxin antigen is supported by the lack of detection of such bands in mutant strain 59A0071 by the monoclo-

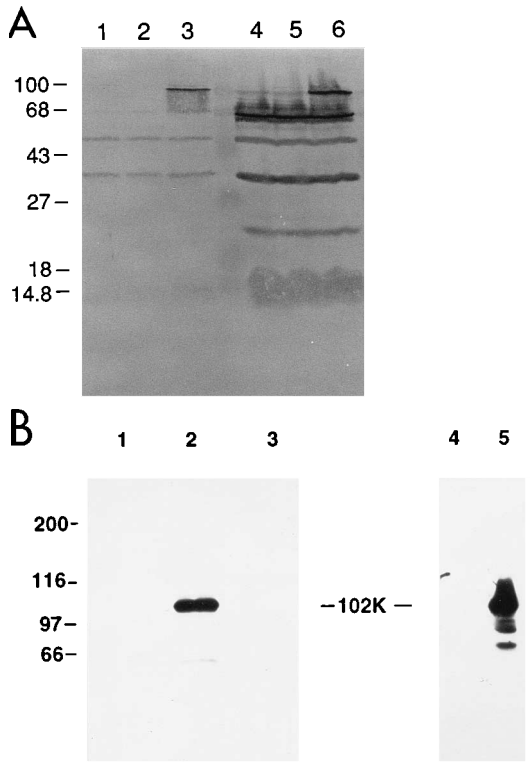

FIG. 3. Secreted and cell-associated antigens in wild-type and mutant *P. haemolytica* strains. The wild-type parent (59B049) and the two mutants were grown at 37°C in BHI broth to a cell density of approximately 8×10^8 cells per ml. The cultures were centrifuged to create a supernatant containing secreted proteins and a cell pellet containing cell-associated proteins. These fractions were processed for electrophoresis and Western blotting as described in Materials and Methods. (A) Western blot with polyclonal anti-PH serum. Lanes 1, 2, and 3, secreted proteins; lanes 4, 5, and 6, cell-associated proteins. Mutant 59B0072 (*lktA2*) is in lanes 1 and 4, mutant 59B0071 (*lktA1*) is in lanes 2 and 5, and parent strain 59B049 is in lanes 3 and 6. (B) Western blot with MAb MM601. Lanes 1, 2, and 3, secreted proteins; lanes 4 and 5, cell-associated proteins. Mutant 59B0071 (*lktA1*) is in lanes 1 and 4, parent strain 59B049 is in lanes 2 and 5, and mutant 59B0072 (*lktA2*) is in lane 3. Molecular mass standards (in kilodaltons) are shown at the left of each panel.

nal, leukotoxin-specific antibody (Fig. 3B, lane 4). In some blots with anti-PH serum, a band slightly larger than the leukotoxin (110 to 120 kDa) was seen in cell supernatants. This band is not seen when antileukotoxin serum or the MAb is used. It thus appears that there is no leukotoxin protein outside or within the cells in either mutant.

There appear to be no other changes to antigens identified with the anti-PH serum in either of the two mutants (Fig. 3A). In particular, two antigens (50 and 35 kDa), which are found in both supernatant and cell-associated fractions, were unaffected. Similarly, several bands that were seen only in the cell-associated fraction were present in similar amounts in parent and mutant strains.

Analysis of the *lkt* locus in the mutants. Genetic mapping is not yet possible for *P. haemolytica*, and so localization of the mutation responsible for the leukotoxin defect must be determined by directly characterizing the DNA in the mutants. One plausible explanation for the lack of detectable leukotoxin is that the gene has been deleted or rearranged. Most types of such gross rearrangements would be easily detected by Southern blot analysis. Restriction enzyme digestion was therefore used to determine whether there were any rearrangements in the *lkt* locus. Initially, a double digestion with the enzymes *Not*I and *Asc*I was performed and the DNA was analyzed by the CHEF variation of pulsed-field gel electrophoresis. These enzymes cut the *P. haemolytica* genome into only about seven fragments, ranging in size from approximately 50 to 800 kb. We observed that the 59B0071 mutant had the same digestion pattern as did the 59B049 parent, while the 59B0072 mutant differed in several bands (data not shown). It is apparent that the mutagenesis resulted in genomic rearrangements in the 59B0072 mutant, probably accounting for its slower growth

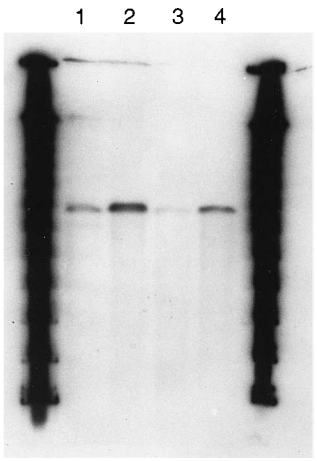

FIG. 4. Pulsed-field gel electrophoresis of wild-type and mutant *P. haemolytica* strains. DNA from wild-type and mutant strains was doubly digested with *Not*I and *Asc*I restriction enzymes and prepared for electrophoresis as described in Materials and Methods. The CHEF gel was run at a ramped pulse time ranging from 5 to 35 s. DNA from this gel was transferred to a membrane and probed with radiolabeled DNA from the *lktCABD* cosmid clone as described in Materials and Methods. The blot was then subjected to autoradiography. The outside lanes are markers of phage lambda concatemers (unit length = 48.5 kb). Lane 1, mutant 59B0072; lane 2, mutant 59B0071; lane 3, wild-type strain 59B049; lane 4, wild-type strain PHL101.

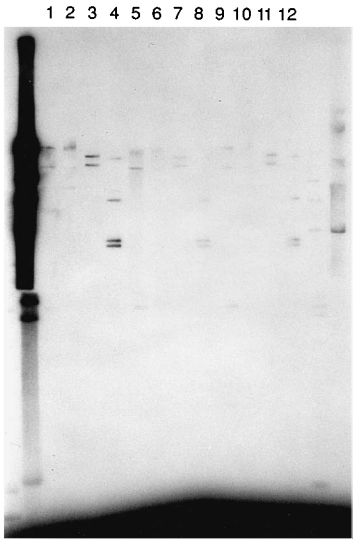

FIG. 5. Southern blot of DNA from wild-type and mutant *P. haemolytica* strains. Shown is an autoradiograph of an agarose gel of digests of wild-type and mutant DNAs probed with the *lktCABD* clone as described in Materials and Methods. Lanes 1 to 4 contain mutant 59B0072, lanes 5 to 8 contain mutant 59B0071, and lanes 9 to 12 contain parent strain 59B049. Lanes 1, 5, and 9, *Xba*I digest; lanes 2, 6, and 10, *Pst*I digest; lanes 3, 7, and 11, *Cla*I digest; lanes 4, 8, and 12, *Bgl*II digest. The outside lanes contain markers. The *Pst*I digest was incomplete.

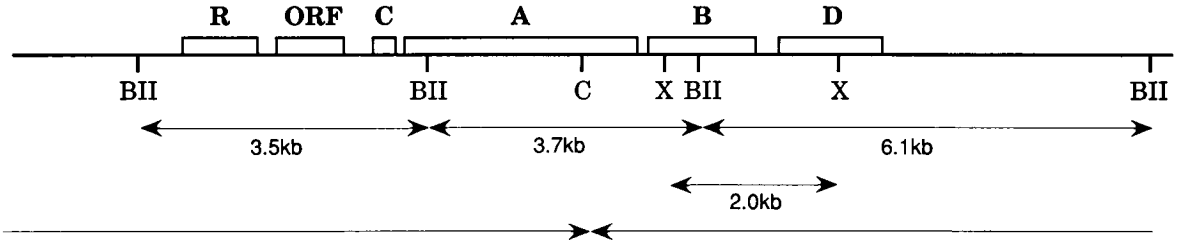

FIG. 6. Restriction map of the *lkt* locus. A map of the *lkt* locus based on its DNA sequence is shown. Gene abbreviations: C, *lktC*; A, *lktA*; B, *lktB*; D, *lktD*. ORF (open reading frame) and R (regulatory locus) represent regions of the *lap* locus (19). Restriction enzyme cleavage site abbreviations: BII, *Bgl*II; C, *Cla*I; X, *Xba*I.

rate. We next examined the fragment that contains the *lkt* locus to determine if the leukotoxin-negative phenotype was due to a large deletion. The pulsed-field gel was blotted and probed with a cosmid that contains a 20-kb insert that includes the entire *lkt* locus (19). As shown in Fig. 4, the fragment that contains the *lkt* locus was the same in the parent and the two mutant strains, despite the differences in restriction fragments between 59B0072 and the other two strains. Also shown in Fig. 4 is the pattern from another *P. haemolytica* strain, PHL101, from which we previously cloned the *lkt* locus. This strain also showed differences from the parental strain used in this study in its genomic restriction pattern (35). It is not known whether the differences in the gel patterns are due to plasmids as well as restriction fragment length polymorphisms. It should be noted that differences such as those seen between PHL101 and 59B049 are common when bacteria are examined by pulsed-field gel electrophoresis (35). However, by Southern blot it is apparent that the same-sized fragment contains the *lkt* locus in PHL101. Thus, we conclude that despite the different total genomic patterns in these four strains, there is no gross rearrangement in the *lkt* region in the two mutants.

To look more closely at this region, digestions were performed with restriction enzymes that cut more frequently and the resulting blots were probed with the cosmid. As shown in Fig. 5, the same pattern was seen for the parent and the two mutants. Fig. 6 shows the restriction map of this region, indicating the fragments detected in the blot shown in Fig. 5. It is clear that no gross rearrangement is present in the mutants.

DISCUSSION

We have used a novel procedure to screen for mutant strains of *P. haemolytica* that fail to produce extracellular leukotoxin. Two mutants were found from approximately 30,000 mutagenized colonies, a frequency of about 10^{-4}, which is reasonable for a forward, loss-of-function mutation. Both mutants appear to be totally deficient in the production of leukotoxin, suggesting that they are defective in transcription of the *lktA* gene, that they contain a chain-terminating mutation (nonsense or frameshift mutation) in *lktA*, or that a highly unstable leukotoxin protein that is degraded without accumulating to a detectable extent in the cell is produced. There are a number of other ways by which mutations could cause a loss in extracellular leukotoxin; for example, by reducing its secretion. However, because of the background immunostaining of *P. haemolytica* colonies it is likely that only the most severely leukotoxin-deficient mutants would be picked up by this screen. Hence, mutants that accumulate intracellular leukotoxin, or that secrete reduced amounts of the protein, may not have been distinguishable from the wild type.

NTG was used as the mutagen in these experiments since many less severe mutagens were impracticable because of their tendency to induce prophages (e.g., UV radiation) or because the limited genetics of *P. haemolytica* precluded their use (e.g., transposon mutagenesis). In addition, it was necessary to use a mutagen that was sufficiently powerful to allow the detection of rare mutations (frequency of 10^{-4}) by an untested assay. The unavoidable problem with NTG mutagenesis is the potential for creating multiple mutations. NTG acts at exposed regions of replication forks and creates clusters of base changes in such regions. In the vicinity of the *lktA* gene are the genes for secretion and activation of leukotoxin, as well as an operon whose regulation appears to be related to the control of leukotoxin production (16, 18, 19). Although it is not known if the *lktA* gene is itself altered in these strains, this appears to be the most likely explanation for the lack of detectable protein. Southern blot analysis indicates that there is no gross rearrangement of this region of the genome in the mutants, and the total genomic restriction map of mutant 59B0071 appears normal. Moreover, at the level of resolution of a Western blot with polyclonal antiserum, it appears that the mutants produce the same antigenic profiles as does the wild-type strain, with only the leukotoxin antigen missing. Thus, although we cannot exclude the possibility that mutant strain 59B0071 contains mutations separate from that affecting leukotoxin, the results presented suggest that no major additional defects occur in this strain.

The leukotoxin-negative mutant 59B0071 described here should prove useful in a number of applications. In an accompanying paper (27), we describe the virulence of strain 59B0071 in animal models of pasteurellosis. In addition, because the mutants show no leukotoxin-derived antigenic bands on Western blots or other protein gels, they can be used for the identification of other antigenic species that would otherwise be obscured by bands derived from the leukotoxin protein or which could previously not be shown to be independent of the leukotoxin. Moreover, these mutants can be used in investigations of the interaction of *P. haemolytica* with leukocytes or other cell types, studies which are currently hindered by the cytolytic activity of the leukotoxin.

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