Inactivation of *Pasteurella* (*Mannheimia*) *haemolytica* Leukotoxin Causes Partial Attenuation of Virulence in a Calf Challenge Model

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The leukotoxin of *Pasteurella* (*Mannheimia*) *haemolytica* is believed to play a significant role in pathogenesis, causing cell lysis and apoptosis that lead to the lung pathology characteristic of bovine shipping fever. Using a system for Cre-*lox* recombination, a nonpolar mutation within the *lktC* transacylase gene of the leukotoxin operon was created. The *lktC* locus was insertionally inactivated using a *loxP-aph3-loxP* cassette, and then the *aph3* marker was excised from the chromosome by Cre recombinase expressed from a *P. haemolytica* plasmid. The resulting *lktC* strain (SH2099) secretes inactive leukotoxin and carries no known antibiotic resistance genes. Strain SH2099 was tested for virulence in a calf challenge model. We inoculated 3×10^8 or 3×10^9 CFU of wild-type or mutant bacteria into the lungs of healthy, colostrum-deprived calves via transthoracic injection. Animals were observed for clinical signs and for nasal colonization for 4 days, after which they were euthanized and necropsied. The lower inoculum (3×10^8 CFU) caused significantly fewer deaths and allowed lung pathology to be scored and compared, while the 3×10^9 CFU dose of either the wild-type or mutant was lethal to ≥50% of the calves. The estimated 50% lethal dose of SH2099 was four times higher than that of the wild-type strain. Lung lesion scores were reduced twofold in animals inoculated with the mutant, while clinical scores were nearly equivalent for both strains. The wild-type and mutant strains were equally capable of colonizing the upper respiratory tracts of the calves. In this study, the *P. haemolytica* lktC mutant was shown to be less virulent than the parent strain.

Pasteurella (*Mannheimia*) *haemolytica* serotype A1 is the primary bacterial agent of bovine pneumonic pasteurellosis, or shipping fever. This multifactorial fibrinonecrotizing pneumonia may be triggered by viral infection, overcrowding, stress, or immunosuppression, which allows the normally commensal *Pasteurella* bacterium to gain access to the lower respiratory tract, where it becomes pathogenic (13). Leukotoxin has long been thought to be the primary virulence factor of *P. haemolytica*, and vaccine development studies have focused on the leukotoxin as an important protective antigen (6).

The *P. haemolytica* leukotoxin (LktA) is a calcium-dependent cytotoxin that is a member of the RTX (repeats in toxin) family. This family includes the *Escherichia coli* hemolysin (HlyA), the *Bordetella pertussis* adenylate cyclase/hemolysin (CyaA), and the *Actinobacillus pleuropneumoniae* Apx toxins (44). *P. haemolytica* leukotoxin is species specific and has cytolytic activity against ruminant lymphoid cells (18, 34). LktA is also weakly hemolytic (25). Though the leukotoxin can bind to cells from a variety of species (39), cytolysis requires a specific interaction with the lymphocyte function-associated antigen 1, or β_2-integrin, on the target (1, 21). At high concentrations, the toxin creates pores in the cell membrane that lead to cell swelling and lysis (5). At sublytic concentrations, the toxin activates neutrophils (8), induces inflammatory cytokine production (48), invokes cytoskeletal changes, and causes apoptosis (37, 38). Like other members of the RTX family, the leukotoxin is expressed from an operon that encodes the protoxin and a transacylase (LktC) that is required to convert the protoxin to an active form (16). Inactive leukotoxin has neither cytolytic nor apoptotic activity in vitro (10, 38, 40), though the inactive toxin can be secreted from bacterial cells (10), and inactive pro-LktA binds with high affinity to target cells (39).

In addition to the in vitro activities described above, the role of leukotoxin in pneumonic disease has been examined in vivo. When instilled into the bovine lung, partially purified leukotoxin was shown to cause cytopathic changes in bovine alveolar macrophages (47), and leukotoxin has been found physically associated with membranes of degenerating macrophages and neutrophils in the alveoli (46). In an attempt to further clarify the role of leukotoxin in bovine pasteurellosis, leukotoxin-negative strains have been constructed and tested for virulence in calves. In one study, a genetically uncharacterized leukotoxin mutant, created by chemical mutagenesis (4), caused reduced mortality and smaller lung lesions than the wild type when inoculated intratracheally (29). In another study, a *lktA* deletion mutant was tested using bacteria delivered endobronchially (41). A similar reduction of mortality and lung lesions was observed. In the latter study, the authors concluded that the mutant "revealed significant reduction in virulence" (41). Based on these studies, we hypothesized that a *P. haemolytica* strain that lacked the *lktC* gene would express and secrete

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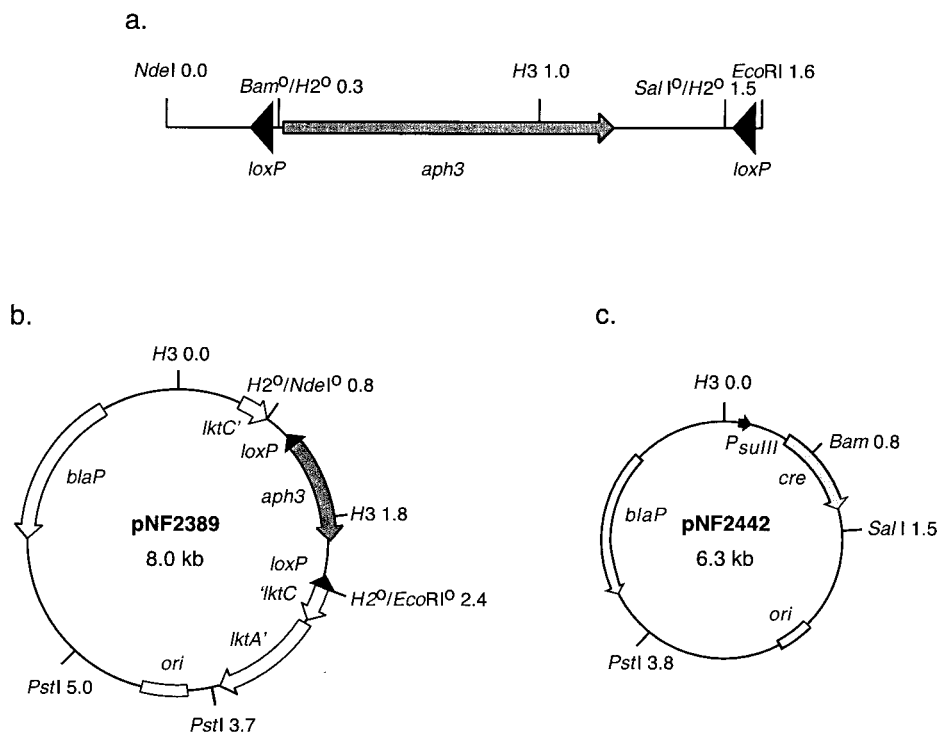
a.

b.

c.

FIG. 1. (a) Abbreviated restriction map of the *loxP-aph3-loxP* cassette. (b) Restriction map of plasmid pNF2389 used for allelic exchange at the *lktC* locus. (c) Restriction map of the *P. haemolytica* plasmid that expresses Cre recombinase under control of the sulfonamide promoter, P$_{sul}$II. Abbreviations: *Bam*, *Bam*HI; *H2*, *Hin*dII; *H3*, *Hin*dIII.

inactive leukotoxin and that such a mutation should cause significant attenuation of the organism. Unlike previous mutants, a *lktC* strain should express all protective epitopes, including those carried on the leukotoxin, and such a strain could be useful as an attenuated live vaccine.

In a prior report, we developed a method for site-specific chromosomal mutagenesis and created a *lktC* strain by inserting a nonpolar promoterless chloramphenicol acetyltransferase gene into *lktC* on the *P. haemolytica* chromosome (10). Since a chloramphenicol-resistant strain is not acceptable as a live vaccine candidate, we sought to develop a *lktC P. haemolytica* strain that lacked a resistance marker. Though we had attempted to create unmarked mutations in *P. haemolytica* using counterselections such as *sacB*/sucrose and tetracycline sensitivity, we were unsuccessful. We turned, then, to the use of site-specific recombination systems that promote precise and efficient excision between two directly repeated sequences that flank a selectable marker. Systems such as Cre/*lox* (36), FLP/*FRT* (2), and λInt/*att* (22) have been used to create unmarked mutations in bacteria, yeast, and higher eukaryotes (20). Among systems for site-specific recombination, the phage P1 Cre/*lox* system seemed to be the most suitable for genetic manipulation of *P. haemolytica* since it requires the activity of only one enzyme, Cre recombinase. In addition, Cre-mediated excision leaves behind a single 34-bp *loxP* site, which creates a nonpolar, stable mutation in the target gene (36). We have succeeded in developing the Cre-*loxP* system for site-specific recombination in *P. haemolytica* and have used it to create an unmarked *lktC* strain. This was tested for its ability to cause disease in a calf challenge model of *Pasteurella* pneumonia.

MATERIALS AND METHODS

Bacterial strains, plasmids and growth conditions. *E. coli* strain XL1-Blue (*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* [F' *proAB lacI^{q}ZΔM15* Tn*10*]; Stratagene, La Jolla, Calif.) was used for plasmid propagation and cloning. *E. coli* was grown at 37°C in liquid or on solid Luria-Bertani medium. *P. haemolytica* strain SH789, isolated from the pneumonic lung of a calf, was kindly provided by Glynn Frank (National Animal Disease Center, U.S. Department of Agriculture, Ames, Iowa). Strain SH1217, a plasmid-cured derivative of SH789, was used as the host for genetic manipulations (11). *P. haemolytica* was grown at 37°C in liquid or solid brain heart infusion (BHI; Difco, Detroit, Mich.) or on 5% sheep blood agar plates (Remel, Lenexa, Kans.). The following plasmids were used: pUC4K (Pharmacia, Piscataway, N.J.), pBCKS+ (Stratagene), pHSG-*cre* (3), pBS30 (33), pNF2176 (11), and pNF2232 (10). Antibiotics were used at the following concentrations for *E. coli* and *P. haemolytica*, respectively: ampicillin, 50 and 25 μg/ml; kanamycin, 50 and 25 μg/ml; streptomycin, 20 and 70 μg/ml.

To create the inoculum for the calf virulence trial, *P. haemolytica* strains were grown in Trypticase soy broth (Difco) to an optical density at 600 nm (OD$_{600}$) of 0.6; then 0.1-ml aliquots were plated on sheep blood agar plates and incubated overnight at 37°C. Cells were recovered from the plates by washing with RPMI 1640 medium (Sigma, St. Louis, Mo.) and were resuspended to a final OD$_{600}$ per milliliter of 3.0 or 0.3 in 0.5× RPMI 1640. Aliquots of the inocula were serially diluted in phosphate-buffered saline, and aliquots were plated on sheep blood agar plates to determine the number of CFU per milliliter.

Genetic and recombinant DNA manipulations. Standard recombinant DNA techniques were used (32). Plasmid DNAs were isolated from *P. haemolytica* or from *E. coli* using a Plasmid Midi kit (Qiagen, Valencia, Calif.). Chromosomal DNAs were isolated using a PUREGENE DNA isolation kit (Gentra, Minneapolis, Minn.). Both *E. coli* and *P. haemolytica* cells were transformed by electroporation as previously described (7). To transfer genes between *E. coli* and *P. haemolytica*, we used an ampicillin-resistant (Ap^r) *P. haemolytica-E. coli* shuttle vector, pNF2176, derived from a native streptomycin-resistant (Sm^r) plasmid, pYFC1 (11). All shuttle plasmids used in this work are derivatives of pYFC1 and are therefore incompatible. This property allows selective heteroplasmid segregation. Oligonucleotides SH117 (TTTTGTTTAATTTCCCTACATTTTGTAT AAC, *lktC* 5′) and SH151 (GCGTCTGTCACCAGACTGCC, *lktC* 3′) were used as primers to amplify DNA flanking the mutagenesis target.

To construct a *loxP-aph3-loxP* cassette, the *aph3* gene from pUC4K was subcloned onto pBS30, which carries two head-to-tail *loxP* sites separated by the yeast *LEU2* gene (33). The 2.5-kb *Bam*HI-*Sal*I *LEU2* fragment of pBS30 was

replaced with the 1.3-kb *Hin*dII fragment carrying the *aph3* gene. The resulting *loxP-aph3-loxP* cassette (Fig. 1a), on a 1.85-kb *Eco*RI-*Nde*I fragment, was inserted into the *Hin*dII site within the *lktC* gene on plasmid pNF2232 (10), to create the mutagenic plasmid pNF2389 (Fig. 1b). Plasmid pNF2389 was electroporated into *P. haemolytica* SH1217, and a double-crossover recombinant, carrying the *loxP-aph3-loxP* cassette at the *lktC* locus, was created as previously described (10).

Plasmid pNF2442 (Fig. 1c) was constructed to express Cre recombinase in *P. haemolytica*. The 1.3-kb *Eco*RI-*Sal*I *cre* fragment of pHSG-*cre* was subcloned onto pBCKS+, which was linearized with the same enzymes. An *Eco*RI-*Kpn*I *cre* fragment was then subcloned onto pNF2176, linearized with *Eco*RI and *Kpn*I, to create pNF2442. On pNF2442, the *cre* gene is expressed under control of a *P. haemolytica* P$_{sulII}$ promoter located 5' of the *Eco*RI site. Following excision of the *loxP-aph3-loxP* cassette from the chromosome of SH2040, the Cre plasmid was cured using novobiocin as previously described (11).

Western blotting and leukotoxin ELISA. Leukotoxin production by *P. haemolytica* strains was assessed by Western blotting as previously described (15). Antigens were detected using polyclonal bovine convalescent serum. Antileukotoxin antibodies were quantitated by an endpoint enzyme-linked immunosorbent assay (ELISA) using leukotoxin prepared by the method of Vega et al. (42). Fifty micrograms of leukotoxin, suspended in 50 μl of blocking buffer (10 mM Tris [pH 7.6], 0.9% NaCl, 2% milk powder, 0.05% Tween), was bound to each well of an Immulon IV plate (Dynex, Chantilly, Va.) by incubation overnight at 4°C. The plate was washed three times with Tris-buffered saline (TBS; 10 mM Tris [pH 7.6], 0.9% NaCl); then bovine serum, diluted in 50 μl of blocking buffer, was added to the plates. Following a 1-h incubation at 37°C, the plate was washed five times with TBS and then incubated with 50 μl of horseradish peroxidase-conjugated goat anti-bovine immunoglobulin G (IgG; 1/200 dilution in TBS; Kirkegaard & Perry, Gaithersburg, Md.) for an additional hour at 37°C. The plate was washed five times more with TBS and then developed for 10 min at room temperature with 50 μl of 2,2'-azino-di-3-ethyl-benzthiazoline sulfonate (400 μg/ml; Roche, Indianapolis, Ind.)–3% H$_2$O$_2$. The reaction was stopped by the addition of 20 μl of 2% sodium dodecyl sulfate per well, and the absorbance of each well was read at 405 nm.

Virulence trial. Sixty-two 100- to 500-lb, mixed-gender, colostrum-deprived Holstein calves were screened for the presence of antileukotoxin antibodies. Thirty calves, selected as having titers less than 400 (reciprocal of serum dilution at which no reactivity was observed), were transported to the test site 5 days before the beginning of the trial (day −4). Serum samples were collected on days −5, 0, and 4, and antileukotoxin IgG titers were determined by ELISA (Table 1). Beginning on day −2, animals were examined daily for clinical signs, as noted in Table 2, and nasal cultures were collected. Calves were assigned to groups in random fashion. Because some of the animals had been used 1 month earlier for a bovine respiratory synctial virus (BRSV) trial, they were first sorted into three groups based on BRSV status: nonvaccinated but challenged (8 calves; group A), vaccinated and challenged (12 calves; group B), and nonvaccinated and nonchallenged (10 calves; group C). Animals in each BRSV group were then assigned a number using a random number generator, and group assignment (1, 2, 3, or 4) was then determined by consecutive numbering. On day 0, each calf received a 5-ml suspension of *P. haemolytica* bacteria (mutant or wild type) delivered transthoracically into each lung as described by Panciera and Corstvet (27). Calves that died before the end of the trial (day 4) were necropsied, and lungs and trachea were removed for subsequent analyses. On day 4 (96 h postinoculation), surviving calves were euthanized and necropsied. The lungs were removed, and the focal injection site lesions, edema, pleuritis, and inflammatory spread were scored by the method of Panciera et al. (28). Mean clinical and lesion scores were analyzed by the Student's *t* test, and 50% lethal doses (LD$_{50}$s) were calculated by the method of Reed and Muench (30).

Lung tissue was cultured for bacteria by sampling the cut surface of a lesion with a sterile cotton swab and then by culturing the inoculum on sheep blood agar plates overnight at 37°C. Nasal swabs were similarly cultured on blood agar. Presumptive *P. haemolytica* isolates were identified and single-colony purified. Bacteria were characterized with respect to oxidase activity, mannitol and sucrose fermentation, and hemolytic phenotype and by PCR analysis of leukotoxin locus, using primers SH117 and SH151. Bacterial serotyping was performed using type-specific antisera provided by Glynn Frank by the slide agglutination method (14).

RESULTS

Construction of antibiotic-sensitive *lktC* strain SH2099. To create an unmarked *lktC* strain, we first disrupted the *lktC* gene by allelic exchange in *P. haemolytica*, using a plasmid incompatibility system to enrich for the isolation of double crossovers (10). Briefly, the mutagenic plasmid pNF2389 (Fig. 1b), carrying *lktC* insertionally inactivated with the *loxP-aph3-loxP* cassette, was introduced into strain SH1217. The resulting strain was then electroporated with the incompatible Sm^r plasmid, pYFC1. Transformants were pooled and propagated overnight
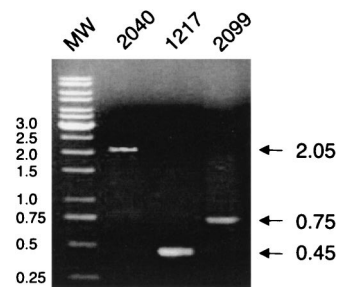

FIG. 2. PCR analysis of the *lktC* locus in wild-type strain SH1217, mutant strain SH2040 (carries the *loxP-aph3-loxP* insertion within *lktC*), and mutant strain SH2099 (contains a single *loxP* site within *lktC*). Positions of DNA molecular weight markers (MW) are reported in kilobases.

in BHI broth containing streptomycin to permit plasmid segregation. To identify isolates where the *aph3* gene had been rescued by allelic exchange at the leukotoxin locus, an aliquot of the overnight culture was spread onto sheep blood agar plates containing kanamycin and streptomycin. Double recombinants (Sm^r Km^r Ap^s) were detected by replica plating onto ampicillin plates. Three nonhemolytic Ap^s Km^r strains were identified out of 400 Sm^r Km^r colonies screened, and one, strain SH2040, was chosen for further analysis.

To excise the Km^r marker from the chromosome, we expressed Cre recombinase in *P. haemolytica* on plasmid pNF2442 (Fig. 1c). Plasmid pNF2442 was electroporated into strain SH2040 with selection for Ap^r. Transformed cells were propagated overnight and screened for the loss of Km^r. Approximately 1% of the colonies were Km^s, presumably resulting from Cre-mediated excision of the *aph3* gene. One Km^s isolate, strain SH2099, was cured of pNF2442 and was characterized further.

Verification of the *lktC'-loxP-'lktC* mutation in the chromosome of SH2099. The presence of *loxP* at the leukotoxin locus on the SH2099 chromosome was demonstrated by PCR analysis (Fig. 2) and verified by Southern blotting (data not shown). A pair of *lktC*-specific primers (SH117 and SH151) was used to amplify chromosomal DNA from the SH2040, SH2099, and wild-type SH1217 strains. As illustrated in Fig. 1, the 0.45-kb wild-type amplimer from SH1217 was replaced by a 2.05-kb fragment in SH2040. Excision of the cassette from SH2040 to create SH2099 resulted in amplification of a 0.75-kb fragment, consistent with expectations. The DNA sequence of the 0.75-kb fragment from SH2099 was determined to verify the precise location of insertion (data not shown). The sequence confirmed that the insert contains the *loxP* site plus 300 bp of flanking DNA that was present on the fragment excised from the pBS30 vector (Fig. 1a). The insertion caused a frameshift at codon 76 of the *lktC* gene.

Strain SH2099 secretes inactive leukotoxin. The mutant strains were tested for cytolytic activity against bovine erythrocytes. Both SH2040 and SH2099 were nonhemolytic on blood agar plates, indicating a loss of cytotoxic activity. *trans* complementation with the *lktC* plasmid restored hemolysis to SH2099 but not to SH2040 (data not shown). Cytotoxicity of SH2099 was not quantitated because a nonhemolytic, inactivated leukotoxin, created by a similar nonpolar insertion in *lktC*, had no leukotoxic activity (10). Loss of hemolytic phenotype has been 100% correlated with loss of leukotoxicity both in *P. haemolytica* mutants (10, 25, 41) and in complementation studies in *E. coli* (12, 16). To examine leukotoxin expression and secretion, Western blot analysis of *P. haemolytica* cell lysates and supernatants was performed using bovine polyclonal
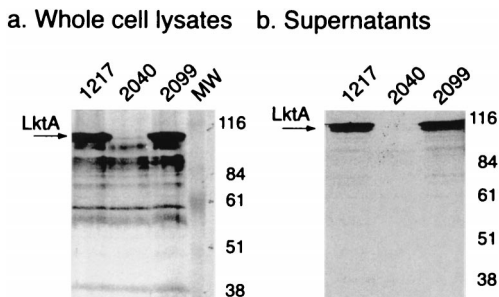
a. Whole cell lysates   b. Supernatants

FIG. 3. Western blot of whole cell lysates (a) and cell-free supernatants (b) of wild-type strain SH1217, mutant strain SH2040, and mutant strain SH2099. Positions of protein molecular weight markers (MW) are reported in kilodaltons.
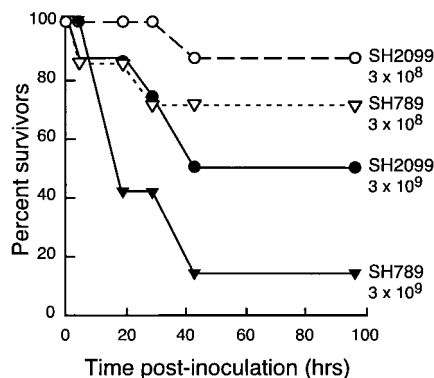

FIG. 4. Calf mortality of wild-type (SH789) and mutant (SH2099) *P. haemolytica* strains when injected transthoracically with 3×10^8 or 3×10^9 CFU/lung.

convalescent serum (Fig. 3). As expected, both SH1217 and SH2099 expressed and secreted LktA at approximately equivalent levels. Strain SH2040, which carries the polar *loxP-aph3-loxP* cassette, does not produce leukotoxin. Thus, the single *loxP* insertion is nonpolar and does not significantly affect downstream leukotoxin expression or secretion in SH2099. The stability of the *loxP* insertion in SH2099 was verified by passaging the strain in BHI broth and plating cells on sheep blood agar plates in an effort to detect hemolytic revertants. No reversion was observed following approximately 100 generations of growth in BHI broth.

Virulence properties of SH2099. The virulence of SH2099 was compared to that of the wild-type parent, SH789, using a transthoracic intrapulmonic challenge-exposure protocol (27). Animals were assigned to one of four groups containing seven or eight animals each, and each lung was injected with either 3×10^8 or 3×10^9 CFU of the wild type or mutant in a 5-ml suspension (Table 1). Following the injections, the calves exhibited depression and labored breathing that persisted for about 8 h. By 4 h postinoculation, two of the calves (one each in groups 2 and 3) had died. Four calves were dead 18 h postinoculation (Fig. 4). Additional animals were found dead at 28 and 42 h; totals are summarized in Table 1. The difference in survival for both the high and low inocula was significant (Fig. 4). At the 3×10^8 CFU inoculum, final survival was 87% for the mutant and 71% for the wild-type strain ($P = 0.03$). At 3×10^9 CFU, 50% of the calves injected with the mutant bacteria survived, while only 14% (one calf) injected with the wild-type survived ($P = 0.005$). LD_{50}s were calculated based on the numbers of deaths in each group. The LD_{50} of SH789 was 7.1×10^8 CFU/lung and the LD_{50} for the SH2099 mutant was 2.9×10^9 CFU/lung. Thus, the mutant appears to be about one-fourth as lethal as the wild type when tested by transthoracic challenge in susceptible calves.

Clinical signs were not significantly different for animals receiving wild-type or mutant bacteria, though scores did correlate with bacterial dose (Table 2). Mean rectal temperatures were slightly elevated in group 3 animals (wild type, 3×10^8 CFU). In contrast, temperatures for calves receiving the larger inoculum demonstrated a significant dip on day 1 postinoculation (data not shown). This reflected a high proportion of animals that were near death following the inoculation on day 0.

Gross lung pathology provided the most rigorous means of distinguishing between the wild-type and mutant strains. A comparison of lung lesion scores for all animals (Table 3) showed lower scores for animals receiving 3×10^8 CFU of mutant versus the wild-type organism ($P < 0.10$). Specific scoring criteria were also compared for calves in groups 1 and 3 that survived the challenge and were sacrificed on day 4 (Table 3). For these surviving animals, focal necrotic lesions were always observed at the injection site, but calves receiving the wild-type bacteria had larger lesions. The edema ($P < 0.05$) and pleuritis ($P < 0.01$) scores were significantly greater in calves receiving wild-type bacteria, and trans- and interlobular extension scores also were higher in these calves than in calves receiving the mutant strain.

All blood cultures were negative, indicating the inoculum was confined to the respiratory tract. Bacteria isolated from nasal swabs and lung tissue were identified as *P. haemolytica*, first by morphology and then by biotyping and serotyping. Serotype A1 isolates were further characterized by antibiotic resistance profile and by PCR to detect the presence of the mutated *lktC* gene. Nasal carriage was monitored to provide information on upper respiratory tract colonization (Fig. 5). Overall, animals inoculated with SH2099 (low dose) had the

TABLE 1. Groups, anti-LktA IgG titers, and survival statistics

Group	Strain	Inoculum (CFU/lung)	No. in group	No. of deaths	Anti-LktA IgG titer[a] (mean ± SD)
1	SH2099	3×10^8	8	1	240 ± 110
2	SH2099	3×10^9	8	4	210 ± 98
3	SH789	3×10^8	7	2	200 ± 110
4	SH789	3×10^9	7	6	250 ± 95

[a] Mean of titers from samples collected on days −5 and 0, reported as the reciprocal of the greatest serum dilution at which a positive reaction was observed by ELISA.

TABLE 2. Clinical scores

Group	Strain	Inoculum (CFU/lung)	No. of survivors	Clinical score[a] (mean ± SD)	
				All calves	Surviving calves
1	SH2099	3×10^8	7[b]	3.4 ± 4.5	1.9 ± 1.6
2	SH2099	3×10^9	4[c]	9.0 ± 5.2	5.8 ± 5.4
3	SH789	3×10^8	5	3.4 ± 4.6	0.8 ± 1.1
4	SH789	3×10^9	1	12 ± 2.0	13

[a] Assigned as follows: Depressed and/or off feed, 1; sneezing and/or coughing, 2; purulent nasal discharge, 1; purulent ocular discharge, 1; respiratory distress, 3; oral and/or nasal mucosal lesions, 4; diarrhea (loose, watery, or bloody stools), 3; moderate rectal temperature elevation (103.5 to 105°C), 3; severe rectal temperature elevation (>105°C), 4 (maximum score, 22).
[b] $P = 0.03$, group 1 versus group 3.
[c] $P = 0.005$, group 2 versus group 4.

TABLE 3. Mean lung lesion criteria and total scores for animals receiving 3×10^8 CFU/lung

Group (strain)	Total lung (all calves)	Score[a] (mean ± SD)					
		Surviving calves					
		Focus	Edema	Interlobular extension	Translobular extension	Pleuritis	Total lung
1 (SH2099)	4.0 ± 6.7[b]	0.4 ± 1.2	0[c]	0.9 ± 1.5[c]	0.3 ± 0.3[b]	0.1 ± 0.4[d]	1.7 ± 1.7[d]
3 (SH789)	9.5 ± 7.6	1.0 ± 1.9	0.6 ± 1.3	2.2 ± 1.8	0.7 ± 0.8	0.9 ± 0.6	5.4 ± 3.7

[a] Determined as described by Panciera et al. (28). Lungs from calves that died before the end of the experiment were not scored for specific lung criteria and were assigned a score of 20.
[b] $P < 0.10$.
[c] $P < 0.05$.
[d] $P \leq 0.01$.

fewest positive cultures, supporting its reduced virulence. Nasal swabs of group 3 animals were culture positive twice as frequently as the group 1 animals, but a number of atypical *P. haemolytica* isolates were also obtained. For example, strain SH789 was recovered from two group 1 nasal swabs, and nontypeable *P. haemolytica* isolates were recovered from calves in groups 2 and 4, but only on day 0. Serotype A2 was found sporadically in nasal swabs of all groups but was not observed in lung lesions. Since *P. haemolytica* A2 is a common pathogen of sheep, it is possible that its source was a lamb barn on the farm where the calf trial was held. Nevertheless, A2 is also a normal part of the bovine upper respiratory flora, and the animals may have been colonized before transport to the trial location. Lung lesions yielded positive cultures for *P. haemolytica*, with the exception of one right and four left lungs in group 1 and two right lungs in group 3. All lung cultures were pure bacterial cultures. Unexpectedly, SH2099 was recovered from the right lung lesions of two group 3 animals. We suspect that these samples were contaminated during sampling or on subculture of postnecropsy samples and believe that the error did not occur during transthoracic inoculation because a single injection was prepared per animal. Finally, two Tc[s] Sm[r] isolates of SH789 were cultured from group 3 and 4 lung samples. These may represent plasmid-cured derivatives of the original inoculum.

DISCUSSION

This is the first report of creation of a defined, unmarked, nonpolar mutation within the genome of *P. haemolytica*. The insertion of a single copy of the bacteriophage P1 *loxP* site within the *lktC* open reading frame caused a nonpolar frameshift mutation that had no effect on downstream expression of the *lktA*, *lktB*, and *lktD* genes. This created strain SH2099, which produces and secretes inactive but antigenic leukotoxin. The *loxP-aph3-loxP* cassette described here should be useful for construction of other such mutations in the members of the family *Pasteurellaceae*. Similar *loxP* cassettes carrying other resistance markers have been constructed (N. D. Fedorova and S. K. Highlander, unpublished data). Placement of a *loxP* site in the *P. haemolytica* chromosome should permit us to create targeted insertions at the *lktC* locus or at other sites where allelic exchange can be accomplished. The system can also be used to create large deletions in the *P. haemolytica* chromosome.

Since leukotoxin is believed to be a critical factor in *P. haemolytica* pathogenesis, we were surprised that inactivation of the toxin caused only a minor reduction in virulence in our calf challenge model. By comparing the strains at different bacterial doses, we established an LD_{50} for the wild-type strain of about 7×10^8 organisms/lung. This reinforces prior studies

that showed that 10^9 CFU was required to reproducibly produce disease when inoculated into the lung (27, 35). The LD_{50} of the mutant strain was about four times higher than the wild-type value (3×10^9 CFU/lung), indicating that the mutant retained significant virulence. Survival curves were most illustrative of the differences between the strains. These curves revealed that a narrow dosage range exists for establishment of *P. haemolytica* pneumonia. In this study, 10^9 CFU/lung generally caused severe morbidity and mortality, while 10^8 CFU/lung failed to induce clinical signs and caused reduced pathological changes. Because of the toxicity of the higher dose, significant differences between the wild type and *lktC* mutant were obscured; at the 3×10^8 CFU dose, however, differences in gross lung pathology were apparent. For calves that survived the challenge, the total lung lesion score was significantly reduced ($P < 0.01$), as were pleuritis and edema scores ($P < 0.05$). Trans- and interlobular extension scores were also reduced, but to a lesser degree. By scoring specific criteria, it appears that presence of active leukotoxin is highly correlated with edema, pleuritis, and inflammation but not with lesion formation.

Our results are similar, but not equivalent, to those reported by Tatum et al., where the virulence of a *lktA* deletion mutant was examined in an endobronchial calf challenge (41). In contrast to our results, 5×10^9 CFU of the $\Delta lktA$ mutant caused no clinical signs of disease. Regardless, the mutant still produced lung lesions, but they were reduced 80% relative to the wild-type parent. Only four calves per group were compared, and an LD_{50} was not calculated. Petras et al. tested the virulence of a chemically induced, leukotoxin-minus mutant in calves and goats (29). Lung lesions created by the mutant
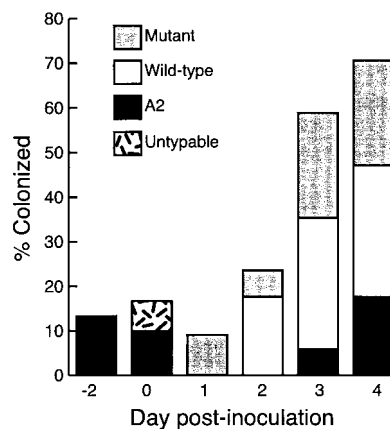

FIG. 5. Nasal colonization of calves with *P. haemolytica*.

strain were reduced 45 to 75% with respect to the wild type; clinical signs were not scored. Since statistical analyses were not applied to the data collected, and because of differences in animal immune status, inoculum preparation, and inoculation route, our study cannot be directly compared to these prior studies. We believe that the 60% reduction in lesion scores that we observed using SH2099 is in line with the level of reduction reported for the leukotoxin-deficient strains and suggests that this represents only partial attenuation of virulence.

The role of other RTX toxins in the pathogenesis of infectious disease has been studied but remains controversial. The bifunctional adenylate cyclase-hemolysin of *B. pertussis* is absolutely required for virulence in infant mice: the LD_{50} of a Cya^- Hly^- Tn*5* insertion mutant was reduced 10,000-fold, though a mutant that was phenotypically Cya^+ Hly^- was only 200 times less virulent (43). This suggests that the adenylate cyclase portion of the molecule is key to *B. pertussis* virulence and that the hemolysin is less critical. *E. coli* hemolysin's role in disease continues to be elusive. Addition of a hemolysin plasmid to a clinical isolate of *E. coli* increased its virulence in a mouse ascending urinary tract model (26). Increased virulence was also observed when $HlyA^+$ *E. coli* were inoculated intraperitoneally into mice (45). Nevertheless, a chromosomal *hlyA* deletion in an enterotoxigenic *E. coli* had no effect on virulence when administered orally to gnotobiotic piglets (24). Thus, HlyA may be most important to extraintestinal disease. The requirement for ApxI expression in porcine pleuropneumonia is also unreconciled. Nonhemolytic mutants of *A. pleuropneumoniae* serotype A5 had an LD_{50} in swine that was 10 times greater than the wild-type value (17). In contrast, a nonhemolytic A2 mutant produced fewer clinical signs than did the wild type but still produced significant lung lesion scores (31). All of these studies suggest that the RTX toxins contribute to the pathogenesis of the disease process but are not the sole, or primary, virulence factors.

Our calf challenge indicates that additional virulence factors in *P. haemolytica* play important roles in lung tissue inflammation and necrosis. It is probable that the early clinical signs that we observed (depression, wheezing) were the due to endotoxemia. Lipopolysaccharide (LPS) has been reported to represent 10 to 25% of the dry weight of *P. haemolytica* bacteria (19), and LPS forms high-molecular-weight aggregates with leukotoxin (23). Since LPS stimulates production of tumor necrosis factor alpha and interleukin-8, leading to inflammation, it is likely that some of the effects that we observed were LPS related. We presume that in the *lktC* strain, unlike leukotoxin knockout strains, leukotoxin-LPS complexes are still formed and secreted. If these complexes potentiate the action of LPS, just as LPS is thought to potentiate the action of RTX toxins (9), then a strain secreting an inactive toxin might well maintain significant virulence. Our findings underscore the need for continued studies of *Pasteurella* pathogenesis. Other factors (i.e., capsule, glycoprotease, and outer membrane proteins) may be found to be critical, and a search for additional virulence factors is in order.

Since *P. haemolytica* is an opportunistic pathogen, it continues to be of importance to examine the roles of stress and viral predisposition in the etiology of shipping fever pneumonia. In the absence of such factors, it can be difficult to produce bovine respiratory disease in experimental animals (35). By using the transthoracic model in colostrum-deprived calves, we were able to recreate *P. haemolytica* bacterial pneumonia without introducing additional factors that would complicate analysis. Nevertheless, the transthoracic inoculation method does not represent the natural mode of infection and fails to allow a test of upper respiratory tract colonization and subsequent descent of the bacteria into the lung.

The potential for the use of strain SH2099 as a live vaccine candidate is diminished by its minimal attenuation. In an earlier trial, using intranasal inoculation of young, colostrum-deprived calves, we were unable to create disease, even with the wild-type organism (D. M. Dusek, N. D. Fedorova, C. Rinehart, and S. K. Highlander, unpublished data). Since high titers of bacteria are required to cause disease (13), strain SH2099 could be tested for its protective antigenicity at lower doses using different routes of inoculation. Smaller doses of the mutant strain, introduced orally or intramuscularly, may yet be protective against shipping fever pneumonia.

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