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

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Received 9 February 2000/Returned for modification 22 March 2000/Accepted 30 March 2000

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.

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inactive leukotoxin and that such a mutation should cause significant attenuation of the organism. Unlike previous mutants, an \( \text{lktC} \) 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 \( \text{lktC} \) strain by inserting a nonpolar promoterless chloramphenicol acetyltransferase gene into \( \text{lktC} \) on the \( \text{P. haemolytica} \) chromosome (10). Since a chloramphenicol-resistant strain is not acceptable as a live vaccine candidate, we sought to develop a \( \text{lktC} \) \( \text{P. haemolytica} \) strain that lacked a resistance marker. Though we had attempted to create unmarked mutations in \( \text{P. haemolytica} \) using counterselections such as \( \text{sacB} \)/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/\( \text{loxP} \) (36), FLP/\( \text{FRT} \) (2), and \( \Lambda \text{Int/att} \) (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/\( \text{loxP} \) system seemed to be the most suitable for genetic manipulation of \( \text{P. haemolytica} \) since it requires the activity of only one enzyme, Cre recombinase. In addition, Cre-mediated excision leaves behind a single 34-bp \( \text{loxP} \) site, which creates a nonpolar, stable mutation in the target gene (36). We have succeeded in developing the Cre-\( \text{loxP} \) system for site-specific recombination in \( \text{P. haemolytica} \) and have used it to create an unmarked \( \text{lktC} \) strain. This was tested for its ability to cause disease in a calf challenge model of \( \text{Pasteurella} \) pneumonia.

**FIG. 1.** (a) Abbreviated restriction map of the \( \text{loxP-aph3-loxP} \) cassette. (b) Restriction map of plasmid pNF2389 used for allelic exchange at the \( \text{lktC} \) locus. (c) Restriction map of the \( \text{P. haemolytica} \) plasmid that expresses Cre recombinase under control of the sulfonamide promoter, \( P_{\text{Sul}} \). Abbreviations: \( \text{Bam} \), \( \text{BamHI} \); \( H2 \), \( \text{HindIII} \); \( H3 \), \( \text{HindIII} \).
replaced with the 1.3-kb HindIII fragment carrying the aph3 gene. The resulting loxP-aph3-loxP cassette (Fig. 1a), on a 1.85-kb EcoRI-NdeI fragment, was inserted into the HindIII site within the lktC gene on plasmid pNF2232 (10), to create the mutagenic plasmid pNF2389 (Fig. 1b). Plasmid pNF2389 was electroporated into $P. \text{haemolytica}$ 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 $P. \text{haemolytica}$. The 1.3-kb EcoRI-SalI fragment of pHSG-cre was subcloned onto pBCKS +, which was linearized with the same enzymes. An EcoRI-KpnI fragment was then subcloned onto pNF2176, linearized with EcoRI and KpnI, to create pNF2442. On pNF2442, the cre gene is expressed under control of a $P. \text{haemolytica}$ P$_{59}$ promoter located 5' of the EcoRI site. Following excision of the loxP-aph3-loxP cassette from the chromosomal DNA of SH2040, the Cre plasmid was cured using novobiocin as previously described (11).

Western blotting and leukotoxin ELISA. Leukotoxin production by $P. \text{haemolytica}$ strains was assessed by Western blotting as previously described (15). Antigens were detected using polyclonal bovine convalescent serum. Anti-leukotoxin 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 five 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 horse-deradish peroxidase-conjugated goat anti-bovine immunoglobulin G (fg.; 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.

**RESULTS**

**Construction of antibiotic-sensitive lktC strain SH2099.** To create an unmarked lktC strain, we first disrupted the lktC gene by allelic exchange in $P. \text{haemolytica}$, using a plasmid incompatible 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 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$_{R}$) were detected by replica plating onto ampicillin plates. Three nonhemolytic Ap$_{R}$ 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 $P. \text{haemolytica}$ 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$_{R}$, presumably resulting from Cre-mediated excision of the aph3 gene. One Km$_{R}$ 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 $P. \text{haemolytica}$ mutants (10, 25, 41) and in complementation studies in *E. coli* (12, 16). To examine leukotoxin expression and secretion, Western blot analysis of $P. \text{haemolytica}$ cell lysates and supernatants was performed using bovine polyclonal

![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.](http://iai.asm.org/article-pdf/19/1/387/4798158/387_0387_Highlander_et_al)
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-apk3-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 × 10^9 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 = 0.05). LD_{50} were calculated based on the numbers of deaths in each group. The LD_{50} of SH789 was 7.1 × 10^9 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 × 10^9 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

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

<table>
<thead>
<tr>
<th>Group</th>
<th>Strain</th>
<th>Inoculum (CFU/lung)</th>
<th>No. in group</th>
<th>No. of deaths</th>
<th>Anti-LktA IgG titer* (mean ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>SH2099</td>
<td>3 × 10^6</td>
<td>8</td>
<td>0</td>
<td>240 ± 110</td>
</tr>
<tr>
<td>2</td>
<td>SH2099</td>
<td>3 × 10^6</td>
<td>8</td>
<td>4</td>
<td>210 ± 98</td>
</tr>
<tr>
<td>3</td>
<td>SH789</td>
<td>3 × 10^6</td>
<td>7</td>
<td>2</td>
<td>200 ± 110</td>
</tr>
<tr>
<td>4</td>
<td>SH789</td>
<td>3 × 10^6</td>
<td>7</td>
<td>6</td>
<td>250 ± 95</td>
</tr>
</tbody>
</table>

*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.

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**TABLE 2.** Clinical scores

<table>
<thead>
<tr>
<th>Group</th>
<th>Strain</th>
<th>Inoculum (CFU/lung)</th>
<th>No. of survivors</th>
<th>Clinical score* (mean ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>SH2099</td>
<td>3 × 10^6</td>
<td>7*</td>
<td>3.4 ± 4.5, 1.9 ± 1.6</td>
</tr>
<tr>
<td>2</td>
<td>SH2099</td>
<td>3 × 10^6</td>
<td>4</td>
<td>9.0 ± 5.2, 5.8 ± 5.4</td>
</tr>
<tr>
<td>3</td>
<td>SH789</td>
<td>3 × 10^6</td>
<td>5</td>
<td>3.4 ± 4.6, 0.8 ± 1.1</td>
</tr>
<tr>
<td>4</td>
<td>SH789</td>
<td>3 × 10^6</td>
<td>1</td>
<td>12 ± 2.0, 13</td>
</tr>
</tbody>
</table>

*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.
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 non-typeable *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 Tcs Smr 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 frame-shift 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 x 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 x 10$^7$ 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$^7$ 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 x 10$^7$ 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 x 10$^9$ CFU of the *ΔlktA* 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

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

<table>
<thead>
<tr>
<th>Group (strain)</th>
<th>Total lung (all calves)</th>
<th>Score (mean ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Focus</td>
<td>Edema</td>
</tr>
<tr>
<td>1 (SH2099)</td>
<td>4.0 ± 6.7$^a$</td>
<td>0.4 ± 1.2</td>
</tr>
<tr>
<td>3 (SH789)</td>
<td>9.5 ± 7.6</td>
<td>1.0 ± 1.9</td>
</tr>
</tbody>
</table>

$^a$ 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 ≤ 0.01.
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 LD₅₀ of a Cya⁻ Hly⁻ Tn5 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 virulence when administered orally to gnotobiotic piglets (24). Virulence factors is in order.

Factors (i.e., capsule, glycoprotease, and outer membrane protein) translocated in 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 higher 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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This study was funded in part by Texas Higher Education Coordinating Board Technology, Transfer and Development grant 004949-037 and by USDA grant 96-35204-3825.

REFERENCES

13. Fedorova, N. D., and S. K. Highlander. 1997. Plasmids for heterologous expression in 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 Pasteurella 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 transheterotic model in colostrum-deprived calves, we were able to recreate Pasteurella haemolytica bacterial pneumonia without introducing additional factors that would complicate analysis. Nevertheless, the transheterotic 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 higher 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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19. Keiss, R. E., D. H. Will, and J. R. Collier. 1964. Skin toxicity and hemody-
namic properties of endotoxin derived from Pasteurella haemolytica. Am. J.
21. Lally, E. T., I. R. Kieba, A. Sato, C. L. Green, J. Rosenboom, J. Korostoff,
1997. RTX toxins recognize a beta2 integrin on the surface of human target
Adaptation of a colorimetric microtitration assay for quantifying Pasteurella
25. Murphy, G. L., L. C. Whitworth, K. D. Clinkenbeard, and P. A. Clinken-
the pathogenicity of pilated pig and also the role of Pasteurella multocidaderived
27. Pace, A. B., M. E. Bauer, A. D. Kent, J. A. Leeds, M. Moayeri, L. B.
Welch, R. A., E. P. Dellinger, B. Minshew, and S. Falkow. 1984. Bovine pneu-
monic Pasteurella haemolytica: effect of vaccination with live Pasteurella
28. Petras, S. F., M. Chidambaram, E. F. Ilyes, S. Froshauer, G. M. Weinstock,
and C. P. Reese. 1995. Antigenic and virulence properties of Pasteurella
Experimental reproduction of acute lesions of porcine pleuropneumonia with
a haemolysin-deficient mutant of Actinobacillus pleuropneumoniae. Vet.
Rec. 129:441–443.
laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold
Spring Harbor, N.Y.
32. Sauer, B. 1987. Functional expression of the cre-lox site-specific recombi-
2096.
35. Stroh, N., and D. Hamilton. 1981. Bacteriophage P1 site-specific recom-
toxin induces bovine leukocytes to undergo morphologic changes consistent
37. Sun, Y., K. D. Clinkenbeard, C. R. Clarke, L. Cudd, S. Highlander, and M.
Dabo. 1998. Pasteurella haemolytica leukotoxin induced apoptosis of bovine
38. Sun, Y., K. D. Clinkenbeard, L. A. Cudd, C. R. Clarke, and P. A. Clinken-
beard. 1999. Correlation of Pasteurella haemolytica leukotoxin binding with
susceptibility to intoxication of lymphoid cells from various species. Infect.
39. Sun, Y., K. D. Clinkenbeard, C. L. Ownby, L. Cudd, C. R. Clarke, and S. K.
Highlander. 2000. Ultrastructural characterization of apoptosis in bovine
61:51–56.
40. Tatum, F. M., R. E. Briggs, S. S. Sreevatsan, E. S. Zehr, S. L. Hsuan, L. O.
isogenic leukotoxin deletion mutant of Pasteurella haemolytica serotype 1:
Adaptation of a colorimetric microtitration assay for quantifying Pasteurella
1564.
a novel toxin that inhibits erythrocyte adenylyl cyclase as virulence factors of
43. Welch, R. A., M. E. Bauer, A. D. Kent, J. A. Leeds, M. Moayeri, L. B.
Regassa, and D. L. Swenson. 1995. Battling against host phagocytes: the
Immunohistochemical localization of Pasteurella haemolytica A1-derived end-
dotoxin, leukotoxin, and capsular polysaccharide in experimental bovine
Morphological and morphometrical analysis of the acute response of the
bovine alveolar wall to Pasteurella haemolytica A1-derived endotoxin and
Pasteurella haemolytica leukotoxin induces expression of inflammatory cyto-

Editor: R. N. Moore