Acylation Enhances, but Is Not Required for, the Cytotoxic Activity of *Mannheimia haemolytica* Leukotoxin in Bighorn Sheep

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***Mannheimia haemolytica* causes pneumonia in domestic and wild ruminants. Leukotoxin (Lkt) is the most important virulence factor of the bacterium. It is encoded within the four-gene *lktCABD* operon: *lkt*A encodes the structural protoxin, and *lkt*C encodes a *trans*-acylase that adds fatty acid chains to internal lysine residues in the protoxin, which is then secreted from the cell by a type 1 secretion system apparatus encoded by *lktB* and *lktD*. It has been reported that LktC-mediated acylation is necessary for the biological effects of the toxin. However, an LktC mutant that we developed previously was only partially attenuated in its virulence for cattle. The objective of this study was to elucidate the role of LktC-mediated acylation in Lkt-induced cytotoxicity. We performed this study in bighorn sheep (*Ovis canadensis*) (BHS), since they are highly susceptible to *M. haemolytica* infection. The LktC mutant caused fatal pneumonia in 40% of inoculated BHS. On necropsy, a large number of necrotic polymorphonuclear leukocytes (PMNs) were observed in the lungs. Lkt from the mutant was cytotoxic to BHS PMNs in an *in vitro* cytotoxicity assay. Flow cytometric analysis of mutant Lkt-treated PMNs revealed the induction of necrosis. Scanning electron microscopic analysis revealed the presence of pores and blebs on mutant-Lkt-treated PMNs. Mass spectrometric analysis confirmed that the mutant secreted an unacylated Lkt. Taken together, these results suggest that acylation is not necessary for the cytotoxic activity of *M. haemolytica* Lkt but that it enhances the potency of the toxin.**

Mannheimia haemolytica is a respiratory pathogen of domestic and wild ruminants (1–3). It is the most important bacterial pathogen of bovine respiratory disease complex, which costs the U.S. cattle industry alone more than $1 billion (4). *M. haemolytica* is also an important pathogen of pneumonia in bighorn sheep (*Ovis canadensis*) (BHS), which is the primary disease responsible for the drastic decline of BHS populations in North America from an estimated 2 million animals in the 1800s to less than 70,000 at the present time (5). Under the experimental conditions, *M. haemolytica* consistently caused 100% mortality in BHS within 2 to 3 days (6–8). The bacterium possesses several virulence factors, including the capsule, outer membrane proteins, lipopolysaccharide, and leukotoxin (Lkt). Based on the fact that Lkt deletion mutants do not cause mortality (6) or cause reduced mortality and milder lung lesions (9, 10), Lkt has been accepted as the primary virulence factor of *M. haemolytica*. The 104-kDa Lkt is absolutely specific for ruminant leukocytes (11). Previously, we have shown that the molecular basis for the ruminant specificity of Lkt rests in its binding to the signal peptide of CD18; the signal peptide remains intact in mature CD18 molecules on ruminant leukocytes, unlike that of nonruminants, which is cleaved (12). Although all ruminant leukocyte subsets are susceptible to Lkt-induced cytolysis, polymorphonuclear leukocytes (PMNs) are the most susceptible, due to their higher level of CD18 expression (13, 14). Lkt-induced PMN lysis and degranulation are the primary causes of the acute inflammation and lung injury characteristic of pneumonia caused by *M. haemolytica*. However, at very low concentrations, Lkt activates target cells to undergo respiratory burst and degranulation. As the concentration of Lkt increases, target cells are induced to undergo apoptosis. At even higher concentrations, necrosis of target cells occurs as a result of membrane damage due to pore formation (15, 16).

Lkt is a member of the repeats-in-toxin (RTX) family of toxins produced by a group of Gram-negative bacteria, including *Escherichia coli*, *Aggregatibacter actinomycetemcomitans*, and *Actinobacillus pleuropneumoniae*. Lkt is encoded by the *lktCABD* operon, where *lktA* encodes the inactive protoxin, *lktC* encodes a *trans*-acylase that adds fatty acid chains to internal lysine residues in the protoxin, and *lktB* and *lktD* encode components of a type 1 secretion system apparatus that, along with the outer membrane protein TolC, secrete the toxin from the cell (17–19). LktC-mediated acylation of LktA protoxin is not required for its expression or secretion (20). It has been reported that LktC-mediated acylation is essential for the biological effects of the toxin, including the induction of apoptosis (20, 21). However, an LktC mutant strain that we (S. K. Highlander) developed previously was only partially attenuated in its virulence in a calf challenge model (22). We reasoned that further elucidation of the role of LktC-induced acylation in the cytotoxic activity of Lkt would be facilitated by characterizing the effects of LktC mutant toxin using target cells that are more susceptible to Lkt than are bovine or ovine cells, which are usually studied in the context of Lkt virulence and activity. PMNs

of BHS are 4- to 8-fold more susceptible to *M. haemolytica* Lkt than are those of domestic sheep (23). Therefore, the objective of this study was to characterize the virulence of the LktC mutant toxin against BHS PMNs *in vivo* and *in vitro*.

MATERIALS AND METHODS

Bacteria, cells, and growth conditions. In this study, *M. haemolytica* strain SH2099 containing a frameshift mutation in the *lktC* gene, developed as detailed by Highlander et al. (22), was used. The parental strain SH1217 (22) was used as a wild-type control in all assays. Both of these bacterial strains are of serotype 1. Bacteria were grown in brain heart infusion (BHI) (Remel, Lenexa, KS) medium at 37°C unless otherwise indicated. To prepare inocula for the animal studies, *M. haemolytica* strain SH2099 was grown on BHI agar plates supplemented with 5% sheep blood (Remel, Lenexa, KS) overnight at 37°C. Bacteria were harvested by scraping with a spreader and were resuspended in BHI broth to obtain an optical density at 600 nm (OD_{600}) of 0.3. The suspension was subcultured for 2 to 3 h at 37°C until the OD_{600} reached 0.8. The bacterial cells were pelleted by centrifugation at $2,400 \times g$ for 18 min and resuspended in colorless RPMI 1640 medium (Life Technologies, Grand Island, NY) to an approximate concentration of 1×10^4 CFU/5 ml. The bacterial CFU count was extrapolated from a calibrated standard growth curve. Bovine lymphoma 3 (BL3) cells (ATCC, Manassas, VA) were grown in RPMI 1640 medium containing 10% fetal bovine serum (Atlanta Biologicals, Flowery Branch, GA).

Animal inoculation. Five adult male BHS, 3 to 5 years of age, from our captive herd at Washington State University were swabbed (nasal and pharyngeal) and bled to identify the presence of Lkt-producing members of the *Pasteurellaceae* and Lkt-neutralizing antibodies, respectively, as described previously (24, 25). Two animals in a group of four were inoculated intranasally with 5 ml of RPMI 1640 medium containing 1×10^4 CFU of *M. haemolytica* strain SH2099 on day 0. The uninoculated animals remained as sentinels. When one of the inoculated animals died on day 4, a new animal was added to the group, and all four animals were inoculated with *M. haemolytica* strain SH2099 as described above on day 103. Nasal swabs, pharyngeal swabs, and blood samples were collected on days 7, 21, 36, 60, 67, and 74 following the first inoculation to evaluate bacterial shedding and transmission and to enumerate Lkt-neutralizing antibody titers, respectively. The animals were housed in Washington State University IACUC-approved facilities and were monitored daily for clinical signs during the course of this experiment.

Production of Lkt. The protocol for Lkt preparation from *M. haemolytica* cultures has been described previously (24). Briefly, bacteria were grown to logarithmic phase in BHI broth, pelleted, and resuspended in twice the BHI broth volume of RPMI 1640 medium supplemented with 0.5% bovine serum albumin (Sigma-Aldrich, St. Louis, MO). After 1 to 1.5 h of growth, the bacteria were pelleted by centrifugation and the supernatant obtained was filter sterilized using a 0.22-μm filter. This supernatant preparation containing Lkt was frozen at −20°C until needed. All Lkt functional assays were performed with the same batch of toxin. In the text below, the terms SH1217 Lkt and SH2099 Lkt refer to the Lkt-containing culture supernatant prepared, as described above, from SH1217 and SH2099, respectively.

Isolation of PMNs from peripheral blood of BHS. PMNs were isolated from peripheral blood of BHS by Ficoll-Paque (Amersham Pharmacia Biotech, Piscataway, NJ) density gradient centrifugation, as previously described (26). PMNs were obtained by hypotonic lysis of the red blood cell pellet.

MTT dye reduction cytotoxicity assay for detection of Lkt-induced cytotoxicity. Lkt activity was quantitated using the MTT [3-(4,5-dimethylthiazoyl-2-yl)-2,5-diphenyl tetrazolium bromide] dye reduction cytotoxicity assay as described previously (24). Briefly, serial 2-fold dilutions of toxins (50 μl/well) were tested in duplicate on aliquots of either PMNs or BL3 cells (5×10^6 cells/ml in 50 μl/well). Toxin cell treatment was carried out at 37°C for 1 h, following which MTT dye was added to the

toxin-treated cells. The plate was then incubated at 37°C for 4 h and 1 h, respectively, for PMNs and BL3 cells. The formazan precipitate that formed was dissolved by the addition of acid isopropanol, and the OD_{540} of each well was measured using an enzyme-linked immunosorbent assay (ELISA) plate reader. The percent cytotoxicity was determined using the following formula: [1 − (OD given by toxin-treated cells/OD given by untreated control cells)] × 100. The 50% toxicity endpoint was defined as the reciprocal of the highest toxin dilution resulting in at least 50% cytotoxicity. The results reported in this study are the mean percent cytotoxicities of three independent measurements.

Detection of Lkt-neutralizing antibody titers. The Lkt-neutralizing antibody titer assay was similar to the MTT dye reduction cytotoxicity assay for detection of Lkt-induced cytotoxicity, with the modification that Lkt was preincubated with serially diluted serum (obtained from the inoculated BHS) for 1 h at 4°C before the addition of cells. In this assay, the toxin dilution that produced 50% toxicity was used. The remainder of the assay was identical to that described above. The percent inhibition of cytotoxicity was calculated as follows: [(cytotoxicity$_{Lkt+medium}$ − cytotoxicity$_{Lkt+serum}$)/(cytotoxicity$_{Lkt+medium}$)] × 100. The Lkt-neutralizing serum endpoint titer of each sample was defined as the reciprocal of the highest test sample dilution resulting in at least 50% neutralization of the toxin.

Flow cytometric analysis. BHS PMNs treated with SH2099 Lkt or SH1217 Lkt were labeled with annexin V and propidium iodide (PI) (Southern Biotech, Birmingham, AL), according to the manufacturer's instructions. Annexin V specifically binds to phosphatidylserine when it is exposed during apoptosis and necrosis, while PI intercalates with the DNA once a cell has been permeabilized due to pore formation. Dual labeling of cells with annexin V and PI is used to distinguish apoptotic cells (only annexin V positive) from necrotic cells (PI positive). Fifty microliters of SH2099 Lkt or SH1217 Lkt was added to 1×10^6 PMNs and incubated for 1 min at 37°C. The cells were pelleted, rinsed once with colorless RPMI 1640 medium, resuspended in cold annexin V binding buffer containing 10 μl of annexin V (Southern Biotech, Birmingham, AL), and incubated for 15 min on ice in the dark. Subsequently, an additional 380 μl of the annexin V binding buffer was added, along with 10 μl of PI. The samples were then immediately analyzed by flow cytometry. Additionally, serial dilutions of SH2099 Lkt were also assayed to evaluate induction of apoptosis. The flow cytometric measurements were performed using a BD FACSCalibur flow cytometer (BD Biosciences, Mississauga, Ontario, Canada), and the data were analyzed using the FCS Express 4 software (De Novo Software, Glendale, CA).

Electron microscopic analysis. Twenty microliters of SH2099 Lkt or SH1217 Lkt was added to 1×10^6 PMNs and incubated for 5 min at 37°C. The cells were pelleted, rinsed once with colorless RPMI 1640 medium, and fixed overnight at 4°C in glutaraldehyde-formaldehyde fixative (2% paraformaldehyde, 2% glutaraldehyde, 0.1 M phosphate buffer). The following day, the cells were rinsed twice in 0.1 M phosphate buffer and fixed overnight at 4°C in 1% osmium tetroxide. The cells were rinsed first with 0.1 M phosphate buffer and then with 0.1 M cacodylate buffer and stored in 0.1 M cacodylate buffer containing 3.5% sucrose until they were imaged. Prior to imaging, the cells were rinsed twice in double-distilled water. Images were taken under a low vacuum setting of 130 Pa using an FEI (Hillsboro, OR) SEM Quanta 200F microscope.

MS analysis. *M. haemolytica* culture supernatant containing Lkt was concentrated using Amicon Ultra-15 Centrifugal Filter Units (EMD Millipore, Billerica, MA) with a membrane nominal molecular mass limit of 50 kDa. The concentrated SH2099 Lkt or SH1217 Lkt was subjected to SDS-PAGE (12% gels) under nonreducing conditions, followed by Western blotting with monoclonal antibody MM605, which is specific for Lkt (24). Bands corresponding to the Lkt protein were excised and trypsinized in the gel before or after acetic anhydride treatment (acetylation). Acetylation has been used in the past to block trypsin cleavage at lysine residues (27). For acetylation, gel slices were first dehydrated with acetonitrile and subsequently treated with bicarbonate buffer containing 5% acetic anhy-
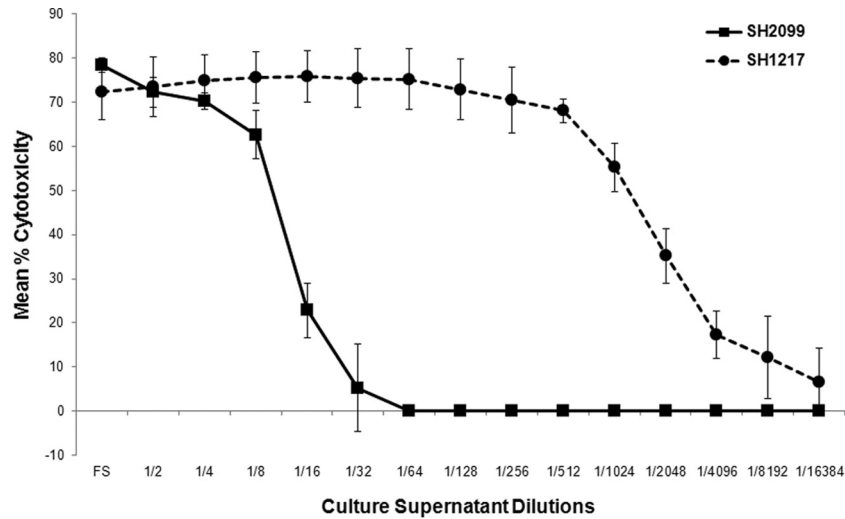
FIG 1 Lkt produced by the LktC mutant strain SH2099 is cytotoxic for BHS PMNs *in vitro*. PMNs from the parent strain, SH1217, and the mutant SH2099 were tested for Lkt-induced cytotoxicity by the MTT dye reduction cytotoxicity assay. The results shown are the means of three independent experiments. The error bars indicate standard deviations of the means.

dride for 5 min. This process of dehydration and acetic anhydride treatment was repeated three more times to acetylate all the lysine residues on Lkt. Subsequently, the gel slices were washed, dehydrated, and dried in a speed-vac prior to addition of trypsin at a concentration of 1 μg/ml for overnight trypsinization at 37°C. The trypsin fragments were analyzed by liquid chromatography-tandem mass spectrometry (LC–MS-MS) as previously described, using an Esquire HCT electrospray ion trap (Bruker Daltonics, Billerica, MA) and an LC Packings Ultimate Nano high-performance liquid chromatography system with minor changes in the LC procedure (28). In MS-MS, a search for peptides containing C_{12}, C_{13}, C_{13} OH, C_{14}, C_{14} OH, C_{15}, C_{16}, and C_{17} fatty acid modifications at lysine residues was performed, using the MASCOT program (Matrix Science Inc., Boston, MA). For the unacetylated peptides, one missed trypsin cut was considered, while for the acetylated peptides, four missed trypsin cuts were considered.

Statistical analysis. A paired Student *t* test was used to compare the cytotoxicities of the Lkt produced by the SH2099 *M. haemolytica* mutant strain on BL3 cells and BHS PMNs. A *P* value of ≤0.01 was considered significant. A repeated-measures analysis of variance (ANOVA) was used to analyze the percent cytotoxicity data. A *P* value of ≤0.01 was considered significant. The statistical analyses were performed using the Minitab 17 software (Minitab Inc., State College, PA).

RESULTS

The mutant SH2099 causes fatal pneumonia in two out of five BHS. One of the two SH2099-inoculated BHS from a group of four BHS died on day 4 postinoculation. The second inoculated animal remained healthy. The two sentinel BHS did not acquire SH2099 from the inoculated animals. When one of the inoculated BHS died, a new BHS was introduced into the group, and all four animals were inoculated with SH2099. Three days later, one more BHS died. In summary, two out of five animals inoculated with SH2099 died. On necropsy, gross pathological examination of their lungs revealed consolidation, hemorrhage, and fibrin deposition. Histopathological examination revealed a large number of necrotic neutrophils (oat cells) in the alveoli, which is pathognomonic of *M. haemolytica*-caused pneumonia. Swabs collected from lesional lung tissue had large numbers of SH2099 bacteria, as revealed by the CFU assay (data not shown). SH2099 was not

isolated from the nasal and pharyngeal swabs collected from the surviving animals throughout the experiment. Only the animal that survived the first inoculation exhibited a Lkt-neutralizing antibody titer of 200 on day 36 postinoculation (data not shown). The other animals did not have significant Lkt-neutralizing antibody titers. None of the surviving animals were euthanized in this study, and hence, no pathological findings are available for them.

Lkt from the LktC mutant SH2099 is cytotoxic for BHS PMNs. The percent cytotoxicities exhibited by Lkt from the parental strain, SH1217, and the mutant SH2099 are shown in Fig. 1. SH2099 Lkt was cytotoxic for BHS PMNs, albeit to a much lower degree than Lkt from the parental strain. To rule out possible contamination with the parental strain, SH1217, the mutant SH2099 was serially diluted and plated. Thirty single colonies of SH2099 were subcultured, and their identities were verified by PCR assay using specific primers (22) targeting the site of mutation. Lkt from each one of the 30 single colonies was cytotoxic for BHS PMNs and not for BL3 cells (data not shown), confirming the fact that the LktC mutant produced a Lkt that was cytotoxic for BHS PMNs, corroborating the *in vivo* finding of 40% mortality caused by the mutant in BHS. In our studies, the toxin in the Lkt preparations (from the SH1217 and SH2099 strains) was not quantified due to its extremely labile nature. In the past, functional Lkt units have been used to quantify the toxin in Lkt preparations. In the current scenario, since there were distinct differences in the functional attributes of the two toxins, quantification of the toxins in this manner would not be meaningful. To normalize the preparations, however, similar numbers of CFU of bacteria, volumes of growth medium, and times of incubation were used in our Lkt preparations.

Lkt from the LktC mutant SH2099 induces necrosis in BHS PMNs. Induction of 40% mortality in inoculated BHS by the mutant and the *in vitro* cytotoxicity of its Lkt to BHS PMNs prompted us to confirm its ability to induce apoptosis and necrosis in BHS PMNs. Dual annexin V and PI labeling was used to determine the occurrence of apoptosis and/or necrosis, using flow cytometry. Following 1 min incubation of BHS PMNs with either the wild-
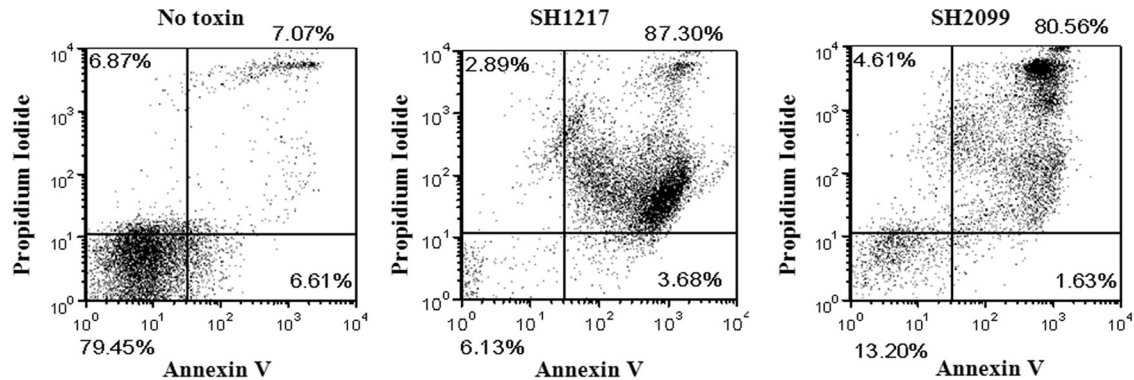
FIG 2 Lkt produced by the LktC mutant SH2099 induces necrosis in BHS PMNs. BHS PMNs were labeled with annexin V and PI following treatment with SH2099 or SH1217 leukotoxin-containing culture fluid. The flow cytometry scatter plots show the percentages of cells that are positive for PI alone (upper left quadrant), for annexin alone (lower right quadrant), and for PI and annexin (upper right quadrant) and negative for PI and annexin (lower left quadrant). The results shown are representative of the results of three independent experiments.

type or the mutant toxin, a massive shift in the cell numbers toward a population of annexin V- and PI-positive necrotic cells was observed (Fig. 2). This pattern did not change even when serially diluted Lkt was incubated with BHS PMNs (data not shown).

Lkt from the LktC mutant SH2099 causes budding and pore formation in plasma membranes of BHS PMNs. Scanning electron micrographs of BHS PMNs treated with SH2099 Lkt or SH1217 Lkt are shown in Fig. 3. Membrane budding indicative of apoptosis and pore formation indicative of necrosis were observed following treatment of cells with either toxin (Fig. 3). At a lower magnification ($\times$5,000), extensive cell-cell adhesion, indicative of leaky cells, was also observed (Fig. 3).

The parental strain, SH1217, Lkt, but not the mutant strain SH2099 Lkt, is acylated at lysine$_{554}$. The lysine residue at amino acid position 554 in *M. haemolytica* Lkt has been predicted to be acylated based on homology to other RTX toxins and an observed decrease in toxin activity following amino acid changes at the residue (29). In this study, we also investigated plausible fatty acid modifications on lysine$_{669}$, which, in alignment with *E. coli* hemolysin, is the closest glycine-lysine pair to the second acylated lysine residue on hemolysin. Hence, we used mass spectrometry to analyze the tryptic fragments of Lkt produced by SH1217 and SH2099 to determine whether lysine$_{554}$ and lysine$_{669}$ are acylated. The mass spectrometric analysis of acylated proteins requires consideration of the fact that the fatty acid chains on the acylated lysine residues (and arginine residues) prevent cleavage carboxy-terminal to modified lysine (and arginine) residues. Furthermore, if tryptic digestion is performed following *in vitro* acetylation, tryptic cleavage occurs following arginine residues, but not after lysine residues, because acetylation of lysine prevents cleavage carboxy terminal to lysine residues. Therefore, tryptic digestion of an acetylated protein would confirm the presence of unacylated lysines in the protein. Hence, acylation of lysine$_{554}$ in Lkt would result in a tryptic peptide spanning amino acids (aa) 550 to 560 containing the acylated lysine$_{554}$. On the other hand, lack of acylation of lysine$_{554}$ would result in tryptic cleavage C terminal to lysine$_{554}$, which would not result in a tryptic peptide spanning aa 550 to 560. As shown in Table 1, tryptic cleavage of Lkt from SH1217, but not SH2099, resulted in peptide 550–560, indicating that lysine$_{554}$ was acylated in Lkt from the parental strain, SH1217, but not in Lkt from the mutant SH2099. The lysine$_{554}$ on this

peptide was modified with the addition of either a hydroxy myristic acid or myristic acid side chain. Moreover, tryptic digestion of *in vitro* acetylated Lkt resulted in the 550-to-565 fragment containing acetylated lysine$_{554}$ from SH2099 Lkt, but not from SH1217 Lkt, further confirming the presence of free lysine$_{554}$ in SH2099 Lkt and acylated lysine$_{554}$ in SH1217 Lkt.

In contrast, the lysine$_{669}$ was not acylated in Lkt from mutant SH2099 or the parent SH1217, as revealed by the absence of the tryptic 670-to-685 fragment. Lack of acylation on both toxins was confirmed by the presence of the tryptic 664-to-685 fragment containing acetylated lysine$_{669}$.

DISCUSSION

Functional *in vitro* studies with an LktA toxin produced from an LktC deletion mutant strain evaluating the production of reactive oxygen metabolites in bovine PMNs and interleukin 8 (IL-8) in bovine alveolar macrophages (BAMs) and release of Ca^{2+} ions in PMNs and BAMs suggested that LktC-mediated acylation was necessary to activate the LktA protoxin (20). Another study with an isogenic LktC mutant showed that the mutant toxin had minimal to no cell cytotoxicity and was incapable of inducing apoptosis on bovine peripheral blood lymphocytes, again suggesting that LktC-mediated acylation is necessary for the activation of the LktA protoxin (21). In summary, while the ability of neutrophils and macrophages to form extracellular traps is not affected by the acylation status of Lkt (30, 31), acylation of LktA is required for IL-8 cytokine production, Ca^{2+} ion release, apoptosis induction, and cytotoxicity in bovine leukocytes. However, the LktC mutant SH2099 previously developed by us (S. K. Highlander) by creating a frameshift mutation in *lkt*C, was only partially attenuated in its virulence against cattle (22). Therefore, it was of interest to us to elucidate the role of acylation in Lkt-induced cytotoxicity. We reasoned that a more susceptible target cell would be a better candidate for elucidating the molecular basis underlying the activation of LktA protoxin by LktC-mediated acylation. The rationale for using BHS in this study is that PMNs of the species are more susceptible to Lkt than those of other ruminants, including domestic sheep and cattle (23).

Our observation that 40% of the mutant-inoculated BHS developed fatal pneumonia was the first indication that acylation may not be absolutely necessary for the toxic activity of Lkt. The
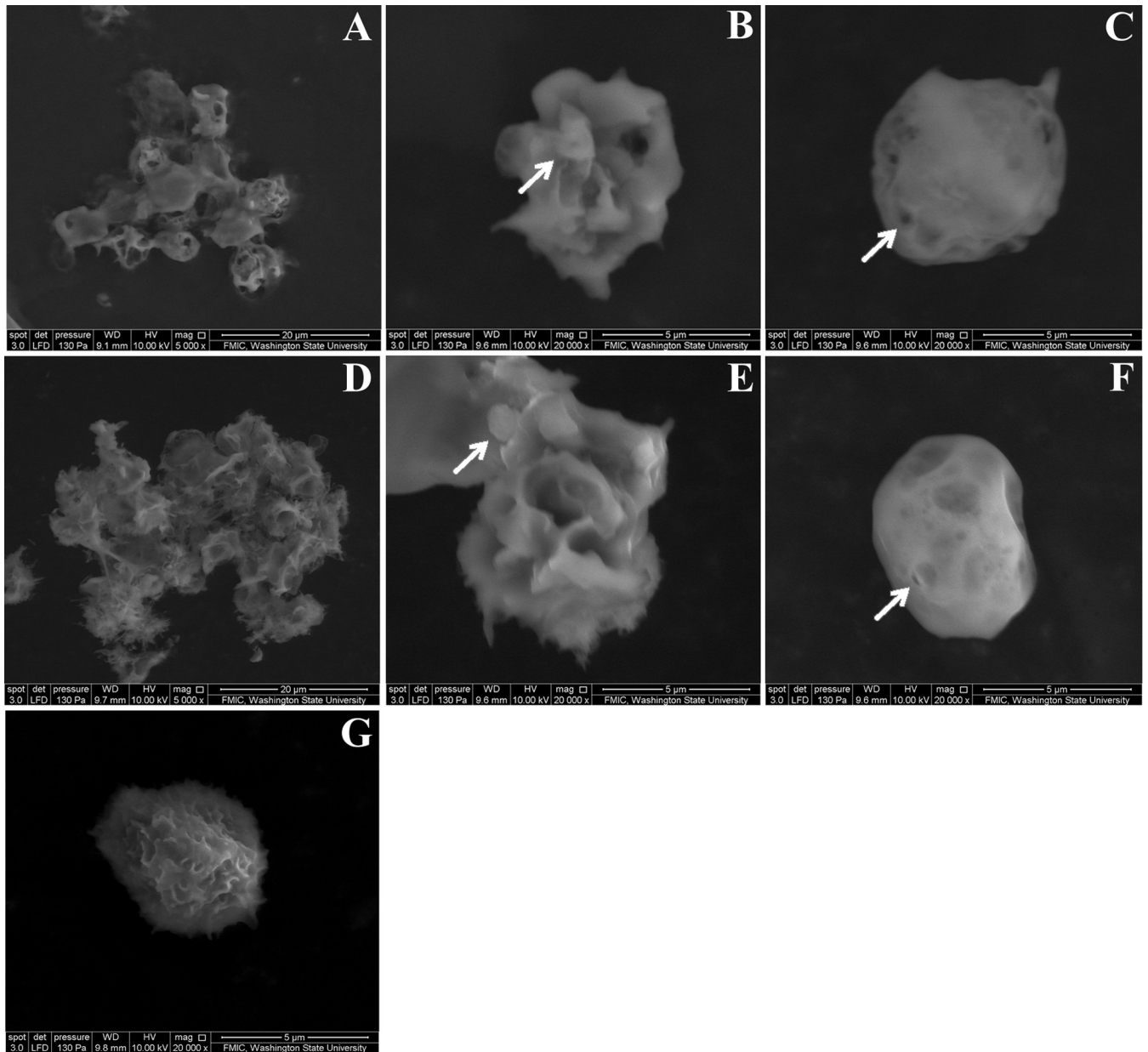
FIG 3 Mutant Lkt-treated cells exhibit pores and blebs on their membranes. Scanning electron microscopy was used to analyze BHS PMNs treated with Lkt from the parent, SH1217 (A, B, and C), or the mutant SH2099 (D, E, and F) strain. Extensive cell-cell adhesion in SH1217 Lkt-treated cells (A) and SH2099 Lkt-treated cells (D) was observed at ×5,000 magnification. Blebs (B and E) and pores (C and F) are indicated by arrows on the membranes of SH1217 and SH2099 Lkt-treated cells (×20,000 magnification). (G) An untreated BHS PMN.

presence of a large number of necrotic PMNs in the lungs of dead BHS supported this notion. It is noteworthy that these animals were inoculated with 1×10^4 CFU, a dose 10-fold lower than the lethal dose of 1×10^5 CFU previously established in our laboratory (25). Due to limited availability of BHS, we could not include control groups of animals that were inoculated with the SH1217 parental wild-type strain or RPMI medium, which could be a possible caveat in interpreting our data. Nevertheless, in our earlier studies involving serotype A1 of *M. haemolytica*, we demonstrated that Lkt of *M. haemolytica* is necessary for the induction of fatal pneumonia in BHS (6). In our animal experiments, it was also of interest to us to evaluate whether the LktC mutant SH2099 would

be attenuated enough to serve as a possible vaccine candidate in BHS, although with the progression of our animal experiments, it became clearly evident that the mutant strain was unsuitable. The surviving animal was reinoculated merely with the intention of administering a booster inoculation.

Lysis of BHS PMNs by the mutant toxin in *in vitro* cytotoxicity assays (Fig. 1) further confirmed the notion that acylation is not necessary for the toxic activity of Lkt. Lkts from all 30 of the single colonies obtained by subculturing the mutant were cytotoxic to BHS PMNs and not to BL3 cells, ruling out the possibility that the mutant was contaminated with the parental strain. These observations prompted us to further characterize the mutant Lkt. Our

TABLE 1 Summary of LC–MS-MS studies on SH2099 Lkt

Lysine position	Leukotoxin	Tryptic fragment observed	Sequence	Lysine modification
554	SH2099	None		
	SH1217	550–560	VQTGKYEYITK	Lys 554 C_{14}
				Lys 554 C_{14} OH
	SH2099 acetylated	550–565	VQTGKYEYITKLNIVR	Lys 554 acetyl
				Lys 560 acetyl
	SH1217 acetylated	550–565	VQTGKYEYITKLNIVR	Lys 554 C_{14}
				Lys 554 C_{14} OH
				Lys 560 acetyl
669	SH2099	670–685[a]	ALHEVTSTHTALVGNR	Not observed
	SH1217	670–685[a]	ALHEVTSTHTALVGNR	Not observed
	SH2099 acetylated	664–685	FVETGKALHEVTSTHTALVGNR	Lys 669 acetyl
	SH1217 acetylated	664–685	FVETGKALHEVTSTHTALVGNR	Lys 669 acetyl

[a] Peptide fragment produced due to lysine$_{669}$.

finding that the mutant Lkt induced necrosis in BHS PMNs comparable to that induced by its wild-type parental strain (Fig. 2) suggested that acylation is not necessary for the toxic activity of LktA protoxin. This view was further supported by scanning electron microscopy on mutant Lkt-treated cells, which revealed pores and blebs on the membranes of dead/dying cells. However, direct comparisons between the two toxins cannot be made, since the toxin preparations were not quantified. Nevertheless, we did use similar numbers of CFU of bacteria, growth medium volumes, and incubation times in our Lkt preparations from either the SH1217 or SH2099 strain to normalize them. Moreover, the same batch of Lkt was used in all the *in vitro* assays. The toxin produced by the SH1217 strain was used only as a positive control for the assay.

Taken together, these observations strongly suggested that acylation is not absolutely necessary for Lkt-induced apoptosis and necrosis of target cells. However, we wanted to confirm that the frameshift mutation that was used to create the mutant in fact abrogated acylation of the Lkt. Mass spectrometric analysis indicated that the toxin produced by the wild-type SH1217 strain of *M. haemolytica* has only one lysine residue (lysine$_{554}$) modified by C_{14} and C_{14} OH fatty acid. This is in contrast to other members of the RTX toxin family, including *A. actinomycetemcomitans* leukotoxin, *E. coli* hemolysin (HlyA), and *Bordetella pertussis* adenylate cyclase toxin-hemolysin (CyaA), where two lysine residues are acylated (32–34). The fact that peptide 550–560 resulted from tryptic cleavage of the parental strain, SH1217, but not the mutant SH2099 confirmed that lysine$_{554}$ was acylated by C_{14} and C_{14} OH fatty acid in Lkt from the parent, but not from the mutant strain SH2099. The presence of peptide 550–565 containing acetylated lysine$_{554}$ in the tryptic digest of acetylated Lkt from the mutant further confirmed the presence of unacylated lysine$_{554}$ in the mutant toxin.

The SH2099 mutant, when cultured on blood agar plates, produces nonhemolytic colonies, in contrast to the parent strain, SH1217 (22). We and others have previously shown that Lkt-induced cytotoxicity of leukocytes is mediated by the interaction of Lkt with CD_{18}, the β subunit of $β_2$-integrins (13, 35). However, similar ligand-receptor interaction has not been identified for the hemolytic activity. Moreover, loss of activity on erythrocytes following abrogation of acylation has been reported for other RTX toxins, including *B. pertussis* toxin CyaA and *E. coli* toxin HlyA (31, 36). Therefore, it is tempting to speculate that the posttrans-

lational modification of LktA by LktC-induced acylation confers hemolytic ability on LktA.

The previous studies that found LktC-mediated acylation necessary for Lkt-induced cytotoxicity were performed with serotype 1 which is the predominant serotype of *M. haemolytica* in bovine pneumonia (37, 38). Serotype 2 is predominant in pneumonia in domestic sheep (37, 38). However, BHS are susceptible to both serotypes 1 and 2 of *M. haemolytica* (6, 25, 39–41). This study was conducted with serotype 1. It is unlikely that different serotypes possess different activation pathways for the Lkt. There are no reports in the literature that suggest otherwise. This is true for other RTX toxins, as well.

In summary, we propose that LktC-mediated acylation of the LktA protoxin is unlikely to confer any qualitative characteristic necessary for the toxic activity of the protoxin. Rather, the added fatty acids, by interacting with the lipids in the plasma membrane, may facilitate the binding of Lkt to its receptor, CD18, thereby enhancing its toxic activity. Thus, acylation is not required for the cytotoxic activity of *M. haemolytica* leukotoxin but enhances its potency.

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